Mitogen-Activated Protein Kinases in Dendritic Cell Maturation And Death

A thesis submitted by

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Abstract

Dendritic cells (DC) sense infection in their microenvironment and undergo a dynamic process of changes in phagocytic capacity, morphology and migratory activity in order to induce optimum T cell immunity. The acquisition of these properties is termed "DC maturation". In this study, key selected aspects of this DC maturation process have been explored.

One aspect of DC maturation is the signalling pathways that DC use to respond to micro-environmental signals. DC respond to conserved microbial structures in their micro-environment via pattern recognition receptors including Toll-like receptors (TLRs). Generally it is thought that this leads to activation of the mitogen-activated protein kinase (MAPK) pathways, p38, ERK, and JNK. JNK and p38 MAPK are also activated by reactive oxygen species, including hydrogen peroxide (H_2O_2), known to be present in the inflammatory DC milieu. Therefore the ability of H_2O_2 to stimulate DC, or modulate their response to TLR ligands such as lipopolysaccharide (LPS) was examined. Activation of JNK, associated with inhibition of tyrosine phosphatases, is linked to induction of DC apoptosis. JNK inhibition partially protected the DC from the pro-apoptotic effects of H_2O_2. Furthermore, H_2O_2 and LPS synergise in inducing JNK activation, and DC apoptosis. These studies suggest that excessive DC maturation, which may induce pathogenic T cell activation may be limited by DC apoptosis.

The second section of the thesis explores the mechanisms leading to MAPK activation upon TLR ligation. Biochemical studies demonstrated that mixed lineage kinase 3 (MLK3), a kinase not previously implicated in DC maturation, was phosphorylated upon TLR3 and 4, but not TLR2 ligation. Studies using a selective MLK inhibitor supported a role for the mixed lineage kinase (MLK) family of MAPK kinase kinase (MAP3K) proteins in regulation of DC signalling, phenotype and cytokine secretion, in response to TLR3 and 4, but not TLR2 ligation. Selective activation of MAP3K may therefore contribute to the heterogeneity and flexibility of DC/pathogen interaction.

A further feature of the DC sensory role is antigen uptake, but the relationship between morphology and phagocytic capacity has not been defined. Therefore a simple and flexible assay to examine this relationship was developed. DC with long dendritic processes were less efficient at antigen internalisation than round DCs.

To Mom, Dad, Sally and James for always supporting me

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The Role of Mixed Lineage Kinases in Dendritic Cell Responses to Different Stimuli

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Table of Abbreviations

APC	Antigen Presenting Cell
ASK1	Apoptosis-Signal Regulating Kinase 1
CAPE	Caffeic Acid Phenethyl Ester
CD40L	CD40 Ligand
CEP	CEP11004
CRD	Carbohydrate Recognition Domain
CRIB	Cdc42 and Rac Interactive Binding Region
CSAID	Cytokine Suppressive Anti-Inflammatory Drug
DC	Dendritic Cell
DC-SIGN	DC-Specific Intracellular Adhesion Molecule-2 Grabbing Non-Integrin
DD	Death Domain
DLK	Dual Leucine-Zipper Bearing Kinase
ECM	Extracellular Matrix
ECSIT	Evolutionarily Conserved Signaling Intermediate in Toll Pathways
EGCG	Epigallocatechin-3-Gallate
ERK	Extracellular Signal Regulated Kinase
FN	Fibronectin
FSC-H	Forward Scatter
GAP	GTPase-Activating Protein
GCK	Germinal Centre Kinase
GEF	Guanine Nucleotide Exchange Factor
H_2O_2	Hydrogen Peroxide
HOSCAR	Human Osteoclast-Associated Receptor
HPK	Haematopoietic Progenitor Kinase
IκB	Inhibitor of Nuclear Factor κB
IFN I	Type I Interferon
IKK	IκB Kinase
IL1	Interleukin 1
IRAK	IL1 Receptor-Associated Kinase
IRF3	Interferon Response Factor 3
JIP	JNK Interacting Protein
JNK	c-jun NH_2-Terminal Kinase
KD	Kinase Domain/ Kinase Dead
LAG3	Lymphocyte Activation Gene 3
LC	Langerhans Cell
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat

LZ	Leucine Zipper
LZK	Leucine Zipper Kinase
ManLAM	Mannose-Capped Lipoarabinomannan
MAP2K	Mitogen Activated Protein Kinase Kinase
MAP3K	Mitogen Activated Protein Kinase Kinase Kinase
MAPK	Mitogen Activated Protein Kinase
MLK	Mixed Lineage Kinase
MMR	Macrophage Mannose Receptor
MSK1	Mitogen and Stress Activated Protein Kinase 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MyD88	Myeloid Differentiation Factor 88
Na_3VO_4	Sodium Orthovanadate
NAC	N-Acetyl Cysteine
NFκB	Nuclear Factor κB
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PD	PD98059
PDC	Plasmacytoid DC
PDTC	Pyrrolidine Dithiocarbamate
PI	Propidium Iodide
PI3K	Phosphatidylinositide-3'-OH Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKR	Protein Kinase R
POSH	Plenty of SH3s
PRR	Pattern Recognition Receptor
PTL	Parthenolide
ROS	Reactive Oxygen Species
RSK2	Ribosomal S6 Kinase 2
SAM	Sterile-α Motif
SB	SB203580
SP	SP600125
SR	Scavenger Receptor
SSC-H	Side Scatter
TAK1	TGFβ-Activated Kinase 1
TBK1	Tank Binding Kinase 1
TCR	T Cell Receptor
TIR	Toll/IL1 Receptor Domain
TIRAP	TIR Domain-Containing Adaptor Protein

TLR	Toll-Like Receptor
TNFα	Tumour-Necrosis Factor α
TPCK	N-Alpha-Tosyl-L-Phenylalanine Chloromethyl Ketone
TRAF	TNF Receptor Associated Factor
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR Domain-Containing Adaptor Inducing IFNß
ZAK	Zipper Sterile-α-Motif Kinase

Chapter 1

Introduction

1.1 Introduction To Dendritic Cells

1.1.1 The role of DC in adaptive immunity

First visualised as Langerhans cells in the skin in 1868, dendritic cells (DC) are now known to function as potent initiators and modulators of immune responses. As professional antigen presenting cells (APC), DC combine the tasks of detecting targets of potential immune responses with the identification and activation of antigen-specific naïve T cells which may exist at extremely low frequency. To perform these diverse roles, DC undergo a maturation process, converting them from specialised antigen seeking cells to potent T cell activators (figure 1.1).

In the immature state, DC are optimised for antigen detection in a number of ways. They exist as a network in the body's periphery, being particularly prevalent at the skin and mucosal sites where pathogen contact is most likely. In this state the DC possess a high endocytic capacity and internalise large volumes of extracellular tissue material either non-specifically via macropinocytosis, or in a specific manner via receptor mediated endocytosis. Internalised material is degraded and presented on MHC class I and II molecules, and this process continues until the DC is induced to mature. The process of maturation is induced by ligation of specific DC receptors by a plethora of different molecular species, including conserved pathogen-derived molecular structures, cytokines, immune complexes, and ligands expressed on the surface of other activated immune cells. The maturation process involves migration from peripheral sites to draining lymph nodes, a reduction in the endocytic capacity, and a concomitant increase in the expression of peptide-loaded MHC complexes. In the lymph node, interaction between MHC class II molecules on the DC and T cell receptors and CD4 molecules on naïve T cells form the antigen-specific central zone of an immunological synapse in which DC-T cell interaction is also facilitated by costimulatory molecules and adhesion molecules.

DC were originally identified on the basis of their morphology (Steinman and Cohn, 1973), and indeed their remarkable morphological capabilities are central to their function in T cell activation. Recent *ex vivo* imaging studies using fluorescently labelled cells has shown that rapid dendrite extensions by DC permits communication with about 5000 T cells per hour (Miller et al., 2004). Mature DC induced formation of stable immunological synapses that last for more than 8 minutes at a time (Miller et al., 2002), sufficient for naïve T cells to receive appropriate stimulation from DC, both via surface molecule interaction and secreted cytokines.

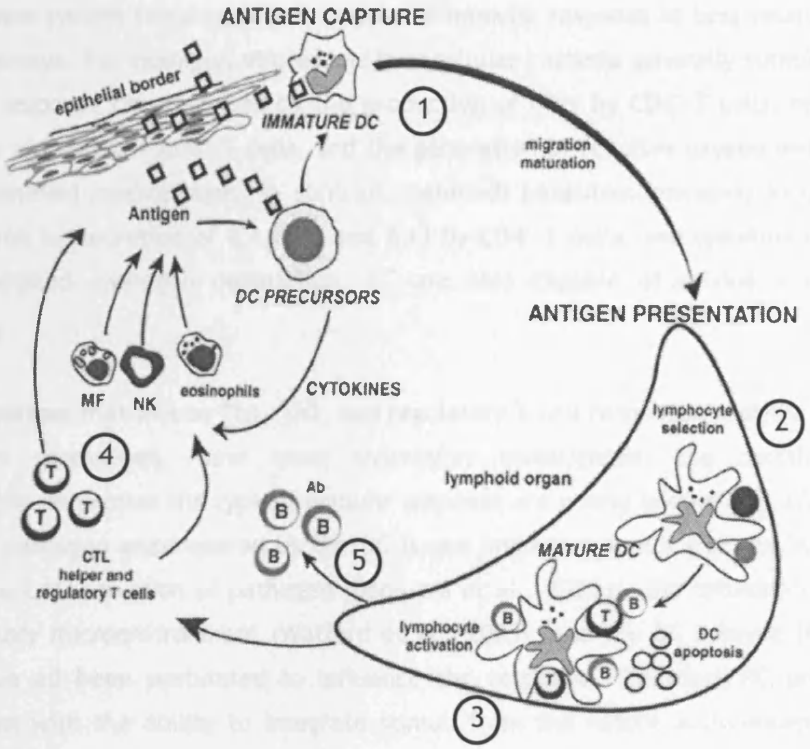

Figure 1.1. The DC life cycle

Immature DCs capture antigen and are induced to mature (1). During this process they migrate to lymphoid organs and display peptide-MHC complexes, which allow selection and thus activation of antigen-specific lymphocytes (2). These activated T cells help DCs in terminal maturation, which allows lymphocyte expansion and differentiation into helper, cytotoxic, or regulatory T cells (3). Activated T lymphocytes then migrate to the infected tissue (4). Helper T cells secrete cytokines, which permit activation of macrophages, NK cells, and eosinophils. Cytotoxic T cells induce lysis of the infected cells. B cells become activated after contact with T cells and DCs and then migrate into various areas where they mature into plasma cells, which produce antibodies that neutralize the initial pathogen (5). After interaction with lymphocytes, DCs die by apoptosis, terminating the response.

Banchereau et al *Annual Review of Immunology* **18**; 767-811 (2000)

In addition to the capacity to activate naïve T cells, DC also have the ability to direct the adaptive immune system towards different types of immune response to best counteract the particular challenge. For example, viruses and intracellular bacteria generally stimulate DC to induce a Th1 response characterised by the production of IFNγ by $CD4^+$ T cells, resulting in the activation of cytotoxic $CD8^+$ T cells, and the generation of reactive oxygen and nitrogen species by activated macrophages. In contrast, helminth parasites commonly induce a Th2 response, driven by secretion of IL4, IL5, and IL13 by $CD4^+$ T cells, and resulting in IgE and eosinophil-mediated pathogen destruction. DC are also capable of driving a regulatory outcome.

Whilst the cytokines that induce Th1, Th2, and regulatory T cell responses, and the nature of the responses themselves, have been thoroughly investigated, the decision-making mechanisms that determine the type of immune response are poorly understood. Clearly, the nature of the pathogen encountered by the DC is one important factor (Re and Strominger, 2001), but, the concentration of pathogen (Boonstra et al., 2003a), the cytokine cocktail of the inflammatory microenvironment (Watford et al., 2003), and the DC subtype (Rissoan et al., 1999) have all been postulated to influence the response. Therefore DC provide the immune system with the ability to integrate stimuli from the nature and concentration of pathogens and inflammatory cytokines, and translate them via antigen presentation, costimulatory molecule expression, and cytokine production into distinct outputs including cytotoxicity, inflammation, tolerance, and memory.

DC that migrate from tissues to lymph nodes have a life expectancy of 2-3 days and can therefore be considered as disposable packets containing a finite amount of antigenic material. During the course of an immune response the nature of the predominant antigenic peptides may be altered, and thus this relatively short life span of mature DC is important in ensuring that the specificity of the activated T cells is relevant to the predominant pathogenic epitopes. Furthermore, in this way, the magnitude of the specific immune response is also matched to the scale of the infection. Apoptosis of DC is therefore an important process in permitting adaptability of the immune response.

Given the importance of DC in controlling specific immunity, and therefore the obvious target for therapeutic intervention that it offers, DC biology is the focus of intense research.

1.1.2 DC subtypes

Two major types of DC exist, classical myeloid DC and plasmacytoid DC (PDC). Unlike the myeloid DC described in the preceding sections, PDC do not possess a dendritic morphology, but rather have a more rounded shape. They also express a different panel of surface proteins. PDC are also characterised by the ability to secrete large amounts of interferon when stimulated with viruses, and to produce this they possess extensive endoplasmic reticulum (Grouard et al., 1997). Like myeloid DC, exposure to pathogen-derived molecules, and in particular those derived from viruses, promotes maturation of PDC to yield cells with dendritic morphology and the ability to activate T cells (Yonezawa et al., 2003).

There is much debate over the developmental origin of the two cell types, which appears to be more plastic than originally thought. The lack of myeloid markers led to the belief that PDC were derived from lymphoid progenitors, however it has recently been shown that murine PDC can be reconstituted from both myeloid and lymphoid precursors (Shigematsu et al., 2004).

Clearly the existence of subtlety different DC subsets optimised to detect and respond to different pathogens is advantageous to the host. These DC populations are also present in different locations; in contrast to the ubiquitous myeloid DC, PDC have only been found in peripheral blood and lymphoid organs (Yoneyama et al., 2004). There have been very few studies into the intracellular signaling of PDC, although PDC have been shown to express different intracellular signaling apparatus to myeloid DC (Izaguirre et al., 2003). For this reason this thesis will focus exclusively on signaling studies undertaken on myeloid DC, and the term 'DC' will refer explicitly to this subset.

1.1.3 DC maturation

Via the ligation of specific receptors, various stimuli induce immature DC to undergo radical phenotypic and functional changes that involve the complete transition from tissue sampling sentinel cells to potent antigen presenters and naïve T cell activators. In this section, the discrete processes of antigen capture and detection, DC migration and T cell stimulation, which together constitute the DC maturation program, will be discussed in more detail.

1.1.3.1 Antigen sampling

Immature DC in peripheral tissues capture pathogens, infected cells, dead cells, or their derived products to be degraded and presented on MHC molecules. Upon maturation, peptide

loading and cell surface delivery of MHC molecules is increased, while internalisation of antigen is reduced. The remarkable capacity of immature DC to internalise antigen is a result of utilising several mechanisms of uptake including receptor-mediated endocytosis, phagocytosis and macropinocytosis. The quantification of the ability of DC to internalise antigen, and in particular, the relationship between this capacity and DC morphology will be investigated in chapter 5.

1.1.3.1.1 Receptor-Mediated Endocytosis

Ligation of specific uptake receptors results in recruitment of cytoplasmic adaptor proteins and ultimately the formation of clathrin coated endocytic vesicles (Slepnev and De Camilli, 2000). DC express a large number of endocytic receptors. In particular, receptors specific for the Fc portion of immunoglobulins, and complement receptors CR3 and CR4, enable DC to internalise immune complexes.

Scavenger receptors (SRs) are glycoproteins that are important for the internalisation of various bacteria. An important scavenger receptor on DC is CD36, which is involved in the uptake of apoptotic bodies (Platt et al., 1998).

A further class of endocytic receptors are the C-type lectins, which bind ligands in a calcium-dependent manner, and possess a carbohydrate recognition domain (CRD) containing four cysteines forming two disulphide bonds. DC express several C-type lectins, including type I multi-CRD lectins such as DEC205 (10 CRDs) (Jiang et al., 1995), and the macrophage mannose receptor (MMR; 8 CRDs), which is the receptor for dextran, a commonly studied ligand in the measurement of DC endocytic capacity (Kato et al., 2000). Type II single-CRD lectins include Langerin (Valladeau et al., 2000) and Dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN) (Steinman, 2000). DC-SIGN is also implicated in cellular adhesion during extravasation and T cell interaction in addition to antigen uptake (Geijtenbeek et al., 2000).

1.1.3.1.2 Phagocytosis

Particulate antigens are internalised by phagocytosis, which is actin-dependent and results in the formation of large intracellular vacuoles. Phagocytosis is generally receptor mediated and facilitated by the same receptors that are required for endocytosis. However, to facilitate phagocytosis, ligation results in actin polymerisation and is not clathrin dependent. Immature DC have been reported to be able to phagocytose most bacteria that have been examined. In gut epithelial monolayers, DC project dendrites through tight junctions and sample bacteria

on the apical side of the epithelia by phagocytosis (Rescigno et al., 2001). DC also phagocytose apoptotic and necrotic bodies via the ligation of various receptors including integrins and CD36.

1.1.3.1.3 Macropinocytosis

Macropinocytosis is a cytoskeleton-dependent type of fluid-phase endocytosis (Sallusto et al., 1995a), and is limited to few cell types including macrophages and DC. Macropinocytosis involves uptake of large vesicles (0.5-3μm) called macropinosomes, which form around areas of membrane ruffling, and is mediated by a rearrangement of the cytoskeleton. DC constitutively macropinocytose, facilitating rapid and non-specific sampling of large volumes of surrounding fluid. Indeed, Sallusto *et al* showed that immature DC can endocytose approximately an entire cell volume within one hour by this mechanism (Sallusto et al., 1995b).

1.1.3.2 Induction of DC maturation

DC maturation is triggered by the ligation of various receptors on the surface of DC by two types of signals. Firstly, direct recognition of conserved structures of invading pathogens (called pathogen-associated molecular patterns (PAMPs)), by a diverse collection of surface and intracellular molecules (termed pattern recognition receptors (PRRs)), provide information to the DC regarding the presence, concentration, and identity of any foreign material (Pasare and Medzhitov, 2005). Maturation can also be induced via the indirect sensing of infection through a variety of sources: inflammatory cytokines and chemokines, internal cellular compounds, ongoing specific immune responses via the recognition of immune complexes, and molecules on the surface of activated immune cells such as CD40L (Moll H., 2003).

Clearly, many of these ligands will exist simultaneously at the site of an infection, and therefore DC maturation is likely to be triggered by the cumulative effect of multiple receptor ligation events. Receptor stimulation induces activation of intracellular signaling pathways leading to gene expression. An important property of DC is therefore the ability to integrate and interpret these multiple signals and translate this information into expression of the appropriate genes resulting in the induction of an optimally targeted specific immune response.

Indeed, the idea of a uniform population of mature DC is an oversimplification, and in fact the precise phenotype of an individual mature DC is likely to reflect that cell's cumulative microenvironmental experiences. Individual mature DC will therefore differ slightly in the cocktails of cytokines secreted and the levels of costimulatory molecules that are expressed, and therefore responding T cells will be activated accordingly. Microarray analyses of gene expression profiles of DC treated with different maturation stimuli support this model. DC uniformly express a set of 'core' response genes in combination with many other genes which are differentially expressed according to nature of the maturation stimulus (Huang et al., 2001; Granucci et al., 2001).

1.1.3.3 Migration of DC

Studies of skin transplantation models and injection of labelled DC demonstrated that DC migrate out of peripheral tissues via the afferent lymphatic vessels. DC maturation stimuli induce DC migration into the T cell area of lymphoid organs. A coordinated program of chemokines controls the migration pattern of maturing DC. Upon stimulation DC upregulate the chemokine receptor CCR7. Accordingly DC acquire responsiveness to CCL19 and CCL21 which are produced by cells in the T cell zone of the lymph node. CCR7-deficient mice have a specific deficiency in DC homing into lymph nodes (Forster et al., 1996).

In addition to controlling DC migration, CCL19 and CCL21 have also been shown to have an anti-apoptotic effect on DC (Sanchez-Sanchez et al., 2004), and CCL19 and CCL21 stimulation of mature DC has also been shown to induce a rapid increase in antigen uptake. This may reflect DC having a final sweep of the infected microenvironment prior to presenting these contents in the lymph node (Yanagawa and Onoe, 2003). Consistent with this interpretation, a transient increase in antigen uptake is also observed upon stimulation with TLR ligands (West et al., 2004).

Even in the absence of obvious maturation stimuli, a small proportion of DC migrate to the lymph nodes, and these DC are always present in afferent lymphatics. The function of these steady-state migratory DC is not clear. They may represent non-tissue restricted 'patrolling' DC. An alternative explanation is that since immature DC express a low but significant level of MHC class II molecules on their surface, which are loaded with self-derived peptides, these cells may be involved in maintaining peripheral tolerance. According to this hypothesis, any T cells that interact with these DC will be self-responders and could cause potential autoimmune complications. However these migratory immature DC will not offer sufficient

stimulation to activate the responding T cells which will presumably be deleted or become anergic (Bonifaz et al., 2002).

1.1.3.3 DC - T Cell interaction

The unique ability of DC to stimulate naïve T cells is probably a result of the high levels of expression of molecules involved in T cell interaction rather than the possession of DC-specific molecules (Inaba et al., 1997). In this section, these DC surface proteins, which are central to the control of T cell activation and are upregulated upon DC maturation, will be discussed.

DC adhesion molecules including β1 and β2 integrins, and members of the immunoglobulin superfamily such as CD54 mediate DC-T cell clustering. Activation of naïve T cells requires three levels of interaction. 'Signal one' involves recognition of MHC class II-peptide complexes by antigen-specific T cell receptors (TCRs). Costimulatory molecules, which are required to sustain T cell activation, constitute 'signal two'. The most important of these for amplification of T cell responses are CD80 and CD86 on DC that ligate members of the CD28 protein family on T cells. 'Signal three' is provided by cytokines and chemokines secreted by DC during T cell interaction, and via this signal DC are able to determine the effector phenotype of naïve T cells. For example, T cells stimulated in the presence of IL12 terminally differentiate into Th1 cells, whereas IL10 leads to production of regulatory or Th2 cells (de Jong et al., 2002). Ligation of OX40 on T cells by OX40L on DC is thought to promote a Th2 outcome (Ohshima et al., 1998). However these soluble and cell-contact-mediated signals which induce DC to bias specific immune responses are numerous, complex and incompletely understood.

Interaction of DC and T cells is not a one-way process. For example, T cells express CD40 ligand (CD40L), which binds to CD40 on DC. This induces increased CD80 and CD86 expression and also potently stimulates the release of cytokines including IL12. The activated T cells also express TRANCE (or RANK ligand), which ligates the RANK protein on DC and has a pro-survival function (Wong et al., 1997). TRANCE is also thought to reinforce the CD40-stimulated induction of cytokine secretion. Furthermore, it has recently been shown that ligation of CD80 and CD86 by CD28 on T cells induces production of IL6 and IL23 by dendritic cells (Orabona et al., 2004). It has been demonstrated in a murine system that one function of IL6 is to make responding T cells refractory to suppression by regulatory T cells (Pasare and Medzhitov, 2003).

1.2 Signaling Pathways Regulating DC Maturation

1.2.1 Introduction

DC control immune responses by detecting pathogens and activating naïve T cells to respond appropriately. As discussed above, the DC maturation process required for the induction of T cell activation involves various DC modifications, including changes in the expression of surface proteins for chemotaxis and T cell interaction, and alterations in DC morphology and migratory capacity. DC maturation is stimulated by the detection of pathogen- and endogenously-derived immunostimulatory molecules via the ligation of specific receptors. This ligand recognition induces the activation of intracellular signaling pathways, of which NF-κB, and the MAPK pathways p38, ERK, and JNK are important examples that will be discussed below. Intracellular signaling pathways are molecular signal transduction systems, which function to transmit signals derived from receptor ligation into cellular outcomes including activation of transcription, cytoskeletal rearrangement, cell migration, etc. Signaling pathways in DC are therefore responsible for translating the recognition of a specific pathogen into the appropriate mature DC phenotype to induce a T cell response. Signaling pathway networks also have the capacity to integrate signals from different receptors and thus allow the DC response to be sensitive to multiple stimuli.

Intracellular signaling pathways therefore offer an ideal target for pharmacological intervention to manipulate the ability of DC to induce specific immunity. Despite this, relatively little is known about the signaling processes underlying DC physiology, and these processes form the focus of the investigations presented in this thesis. As model PAMPs, Toll-like receptor (TLR) ligands are the most widely used maturation stimuli in studies of intracellular signaling pathways in DC. In the following sections, firstly what is known about the mechanism of activation of signaling pathways by TLR ligands will be discussed, and then the signaling pathways themselves and the roles of these pathways in various aspects of DC physiology will be addressed.

1.2.2 Toll-like receptors and induction of intracellular signaling

1.2.2.1 Introduction

Toll receptor was originally identified in *Drosophila* as an essential component regulating dorso-ventral axis formation during embryo development. However, toll-mutant flies were also found to be highly susceptible to fungal infection, suggesting a role for this protein in the

immune system of the insect (Hashimoto et al., 1988; Lemaitre et al., 1996). Mammalian homologues of Toll, termed Toll-like receptors (TLRs) were subsequently identified and found to specifically recognise both pathogen- and endogenously-derived molecular structures. At least 11 mammalian TLRs are known to exist. They show a high degree of sequence similarity to each other and to the IL1 receptor family of proteins in the cytoplasmic domain, which is now termed the Toll/IL1 receptor (TIR) domain. The extracellular regions of the TLRs and IL1Rs are structurally unrelated, however; in contrast to the immunoglobulin superfamily domain of the IL1R, TLRs possess leucine-rich repeats (LRRs), which are thought to facilitate ligand recognition. Individual TLRs elicit specific cellular responses via the differential association between the TIR and various intracellular adaptor proteins (Table 1.1).

Table 1.1 - TLRs; Ligands, localisation, and adaptor proteins

	Ligand	Location	Adaptor(s)
TLR1/TLR2	Triacylated lipopeptides	Plasma Membrane	MyD88, TIRAP
TLR6/TLR2	Diacylated lipopeptides (including Zymosan)	Plasma Membrane	MyD88, TIRAP
TLR3	dsRNA, siRNA	Endosomal	TRIF
TLR4	LPS, sHA	Plasma Membrane	MyD88, TRIF, TIRAP
TLR5	Flagellin	Plasma Membrane	MyD88
TLR7	ssRNA, R848	Endosomal	MyD88
TLR8	R848 in humans, but not mice	Endosomal	MyD88
TLR9	CpG DNA	Endosomal	MyD88
TLR10	Not known, expressed in humans, but not mice	Not known	MyD88
TLR11	Uropathogenic bacteria (mouse), not expressed in humans	Plasma Membrane	MyD88

1.2.2.2 TLR functions

Approximately 10 years before the discovery of TLRs, Charles Janeway hypothesised that signal 2 output by DC was regulated by innate recognition, and this has proved to be correct since all TLR ligands lead to increased expression of CD40, CD80, and CD86 (Janeway, Jr., 1989). However the effect of TLR ligation on DC is not limited to this function. Indeed, it may be argued that the most important DC output modulated by TLR ligands is in fact signal 3 (Reis e Sousa, 2004). For example TLR ligation promotes production of IL12 family members, which promote T cell polarisation (Alber G et al., 2006). Furthermore, different TLRs, or even different ligands of the same TLR, can vary in their ability to modulate the effector T cell bias of the resulting immune response. For example, ligation of TLR3 (de Jong et al., 2002), TLR4 (Boonstra et al., 2003b), TLR7 (Ito et al., 2002), and TLR9 (Hemmi et al., 2000) promote DC to induce a Th1 phenotype. In contrast, some TLR2 ligands induce little IL12 production and skew the immune response towards Th2 (Qi et al., 2003). Based on these observations it

has been suggested that the primary function of TLRs on DC is not to induce maturation *per se* but to provide information about the nature of the insult and thus allow DC to respond appropriately.

In support of this model is the observation that mice deficient for IFNα/β receptors are impaired in their ability to upregulate CD40 in response to TLR ligands. This suggests that DC maturation induced by TLRs is at least partly due to autocrine effects mediated via TLR-induced production of type I interferons (IFN I) and possibly other DC-activatory cytokines such as TNFα (Hoshino et al., 2002). However recent findings have indicated that inflammatory mediators alone are insufficient to induce full DC activation, since in the absence of TLR stimulation, DC are able to promote T cell clonal expansion but do not induce Th1 or Th2 effector differentiation (Sporri and Reis e Sousa, 2005). Clearly both pathogen- and endogenously-derived ligands are required for full DC maturation. This underlines the important message that DC must integrate signals from multiple sources including pathogens and inflammatory mediators, but also chemokines, cell-cell contact, stress molecules, etc, in order to induce the most appropriate immune response.

If the upregulation of costimulatory molecules is not necessarily a direct consequence of TLR ligation, then there are many other functions of DC (in addition to signal 3 modulation), which are TLR mediated. For example TLR4 ligation has been shown to induce upregulation of TLR2, TLR4, and TLR9 (An et al., 2002), presumably as part of an amplificatory system since bacteria contain ligands for all three of these TLRs. Interestingly, however, these TLRs may differentially skew towards Th1 or Th2 and thus this system may also be immunoregulatory depending upon the context. TLR ligation also regulates antigen uptake and processing since TLR ligation in macrophages results in upregulation of expression of scavenger receptors such as MARCO and the mannose receptor (Doyle et al., 2004).

1.2.2.3 Interaction of TLRs with other receptors

To expand the model of DC maturation induced by cytokines in combination with TLR ligands, it should be emphasised that TLRs are not the only microbial recognition receptors. Indeed, pathogens have the potential to ligate many DC receptors and induce intracellular signaling; including C-type lectins, integrins, complement receptors, Fc receptors, and NOD receptors. Indeed, since many pathogens will contain ligands for multiple PRRs, the optimal DC response is likely to be determined by the sum of the intracellular signaling patterns induced by all of these PRRs, in addition to cytokine-induced signals and other inputs.

There is growing evidence for the importance of multiple intracellular signaling events initiated by simultaneous ligation of TLRs and other PRRs in the modulation of the phenotype of the mature DC. For example, in the response of mice to *T. gondii*, DC are activated by ligation of TLR11 (Yarovinsky et al., 2005). However there is also a requirement for a second parasite component, identified as a cyclophilin, which signals via the chemokine receptor CCR5. Consistent with these findings, mice deficient for CCR5 or MyD88 display a marked reduction in IL12 production in response to *T. gondii* (Aliberti et al., 2003).

Another example of TLRs interacting with chemokine receptors is demonstrated by the observation that TLR4 and CXCR4 interact on cell membranes (Triantafilou et al., 2001).

TLR4 can also synergise with NOD2 in the response of myeloid cells to LPS (Nau et al., 2002), and furthermore, TLR4 has long been known to associate with CD14 and MD2 proteins in the initiation of LPS signaling. These accessory proteins were previously thought to concentrate the LPS signal but CD14 has recently been shown to be required for LPS-induced TNFα production (Jiang et al., 2005).

Dectin-1 is a C-type lectin, which binds β-glucans and hence acts as a PRR and phagocytic receptor for innate recognition of yeasts (Brown and Gordon, 2001). Inflammatory signaling in response to zymosan requires a heterodimer of TLR2 and TLR6. It was initially thought that dectin-1 facilitated uptake, while the TLRs were responsible for intracellular signaling and DC maturation. However, in TLR-deficient macrophages, some inflammatory responses are intact suggesting a more complex role for the C-type lectin (Gantner et al., 2003). It has since been shown that dectin-1 signals via syk tyrosine kinase and that syk-deficient DC are unable to produce IL2 and IL10 in response to β-glucans, but do produce IL12 (Rogers et al., 2005).

Another C-type lectin that has been suggested to modulate TLR signaling is DC-specific ICAM3-grabbing nonintegrin (DC-SIGN). The primary function of DC-SIGN is to mediate binding to ICAM2 during extravasation and to ICAM3 during DC-T cell interaction. However DC-SIGN also binds mannose-capped lipoarabinomannan (ManLAM) from *M. tuberculosis*. ManLAM binding has been shown to downregulate IL12 and to stimulate IL10 production by TLR-activated DC (Nigou et al., 2001).

1.2.2.4 TLR signaling

The mechanism of activation of intracellular signaling and MAPKs in particular following ligation of TLRs is incompletely understood. In the following sections, the main features of TLR signaling will be outlined.

The first downstream protein of the TLR signaling cascade to be characterised was Myeloid Differentiation Factor 88 (MyD88), which contains a TIR domain for interaction with the TLR or IL1 receptor, and also a death domain (DD), which facilitates binding to IL1 receptor-associated kinases (IRAKs) (Muzio M et al., 1997). Several additional adaptor proteins have now been identified, including TIRAP (TIR domain-containing adaptor protein) (Fitzgerald K A et al., 2001), TRIF (TIR domain-containing adaptor inducing IFNβ/ TICAM1) (Yamamoto M et al., 2002), SARM, and TRAM (or TICAM2) (Fitzgerald K A et al., 2003). Overexpression of MyD88, TIRAP, or TRIF leads to activation of NF-κB, however the precise roles of all of these adaptors in TLR-mediated signaling, and the differential control of cellular responses is not fully understood.

MyD88-deficient mice are unable to produce inflammatory cytokines such as TNFα, IL6, and IL12 in response to IL1, or any of the TLR2, 7, or 9 ligands, and activation of the NFκB and JNK signaling pathways is not observed in response to ligation of TLRs 2, 7, and 9 in these mice (Kaisho et al., 2001). However, NF-κB and JNK, and many interferon responsive genes, were activated in response to TLR4 ligation in the MyD88-deficient mice, clearly indicating the existence of a MyD88-independent pathway downstream of some TLRs. In this pathway, stimulation leads to the activation of the effector protein interferon response factor3 (IRF3), which induces interferon-responsive genes (Doyle et al., 2002).

TIRAP-deficient mice demonstrate impaired cytokine production, and JNK and NF-κB activation in response to TLR4 ligation. However, TLR4-induced activation of IRF3 is unaffected in TIRAP-deficient, or TIRAP and MyD88-double-deficient macrophages, suggesting that TIRAP is not the factor responsible for the MyD88-independent pathway. In addition to TLR4, TIRAP-deficient mice are retarded in TLR2 responses, but are not affected in their ability to respond to TLR3, 5, 7, or 9 ligands (Horng et al., 2002).

TRIF was first identified as a TLR3-associated molecule, and TRIF-deficient mice are unable to activate interferon-responsive genes in response to TLR3 and TLR4 ligation, suggesting that TRIF mediates the MyD88-independent pathway (Yamamoto et al., 2003). It has been shown that TRIF can associate with TRAF6 and TBK1 (Tank-binding kinase1) in activating the distinct

transcription factors, NF-κB and IRF3, respectively (Sato et al., 2003). There is now evidence, however, that the idea of MyD88-dependent and -independent pathways is an oversimplification, since both pathways are required for some outcomes such as inflammatory cytokine production. LPS in particular exhibits a non-redundant requirement for MyD88, TIRAP, and TRIF, in the induction of inflammatory cytokines, whereas CpG DNA appears to require MyD88 alone (Kaisho and Akira, 2001). On the other hand, MyD88 and TRIF appear to be able to compensate for one another in the LPS-induced production of IFNß (Yamamoto et al., 2003).

TRAM shares sequence similarity with TRIF. The precise role of TRAM is controversial, and it has been suggested that TRAM interacts with various other signalling proteins. Bin et al demonstrated interactions of TRAM with IL1R, TRIF and TIRAP, IRAKs and TRAF6, but not with TLR2 or TLR4 (Bin et al.,2003). Overexpression of TRAM has been shown to induce IRF3 and IRF7 reporter activities (Fitzgerald et al., 2003). In the same study, TRAM was demonstrated to immunoprecipitate with TRIF, IRF3, IRF7, and the IRF3,7 kinases IKKε and TBK1 (Fitzgerald et al., 2003). A TRAM mutant was also shown to block NF-κB activation induced by TLR4, but not other TLRs. Inhibition by siRNA has confirmed that TRAM is not required for TLR3 signaling, but is essential for both the MyD88 dependent and independent arms of the TLR4 signaling pathway (Fitzgerald et al., 2003). In contrast to the immunoprecipitation results, neither TRIF, nor TRAM, bind to MyD88 or TIRAP in yeast two-hybrid experiments (Oshiumi et al., 2003). A TRAM knockout mouse has recently been generated, and is profoundly deficient in LPS responses, but retains TLR3 signaling capacity (Yamamoto et al., 2003).

Figure 1.2 outlines the key features of adaptor usage and intracellular signal propagation by different TLRs.

1.2.2.5 IRAKs

IL1 receptor-associated kinase (IRAK) was originally identified as a kinase that could be coprecipitated with IL1R (Martin et al., 1994). This protein, which we now know as IRAK1, was found to be approximately 90KDa (714 amino acids) and had homology to Pelle - a kinase in *Drosophila* Toll pathway (Cao et al., 1996a).

Four mammalian IRAK proteins have now been identified, IRAK1, -2, -4, and -m. The importance of the IRAK family members in TLR-mediated signaling pathways was first demonstrated in IRAK1 knockout mice, which showed partial inhibition of IL1- or LPS-induced

NF-κB, p38 and ERK activation. In particular, the inhibition was reduced at high stimulation (Swantek et al., 2000). However, IRAK4 knockout mice showed almost complete inability to respond to TLR2, -3, -4, or -9 ligands (Suzuki et al., 2002).

All IRAKs and Pelle consist of an N-terminal DD, which facilitates binding to the adaptor proteins, and a central Kinase domain (KD), which contains 12 subdomains and resembles typical catalytic domains of serine/ threonine kinases. Only IRAK1, IRAK4 and Pelle have catalytically competent kinase domains due to the presence of a critical aspartate residue in subdomain VIb, whereas in IRAK2 and IRAKm this aspartate is an aspartamine or serine residue respectively, resulting in loss of function.

IRAK1, -2, and -m but not IRAK4 or Pelle contain a C-terminal region with no sequence similarity to any other proteins. This region facilitates the binding of IRAK and the downstream effector protein TRAF6 via a conserved Q X P X E motif (Ye et al., 2002; Darnay et al., 1999). The IRAKs must be phosphorylated to permit this interaction, and this is facilitated by IRAK4, which is the closest human homolog to Pelle, and functions as an IRAK kinase. This was determined primarily by the observation that kinase-deficient IRAK1 can be phosphorylated by IRAK4, but not vice-versa, and furthermore transfection of IRAK4 was insufficient to restore IL1-induced NF-κB activation in IRAK1-deficient HEK293 cells (Li et al., 2002). Unlike other IRAK proteins, IRAK4 does not heterodimerise with other IRAK molecules, although stimulation with IL1 induces a stable association between IRAK1, IRAK4 and TRAF6 (Li et al., 2002).

IRAKm can bind both MyD88 and TRAF6, and can heterodimerise with both IRAK1 and IRAK2. IRAKm knockout mice exhibit increased TLR/IL1R signaling, suggesting a negative role for IRAKm. This idea is supported by the observation that IRAKm-deficient macrophages produce higher levels of cytokines in response to TLR ligands, and IRAKm knockout mice exhibit greater intestinal inflammation following salmonella challenge. Interestingly, the most pronounced effects occurred in response to CpG DNA - suggesting TLR-specific roles for IRAK proteins (Kobayashi et al., 2002).

IRAK4 is required for the recruitment of IRAK1 to the receptor complex. Reconstitution of IRAK4-deficient cells with wild-type IRAK4 is capable of restoring IL1 responses (Lye et al., 2004). Interestingly, reconstituting with a kinase-deficient IRAK4 also partially restores IL1 responses, and in a separate study, Qin *et al* showed that the kinase activity is dispensable for IL1 signaling (Qin et al., 2004). These studies suggest that IRAK4 is capable of transmitting

signals via phosphorylation of substrate proteins, and through kinase-independent protein-protein interactions. It was previously thought that phosphorylation of IRAK1 by IRAK4 increases its catalytic activity resulting in multiple autophosphorylation events. However it is also possible that IRAK4 induces IRAK1 autophosphorylation merely by direct protein-protein interaction; therefore the IRAK1 kinase function of IRAK4 may in fact be replaced by that of IRAK1 itself; the eventual goal of IRAK1 hyperphosphorylation being achieved regardless.

Hyperphosphorylated IRAK1 has a reduced affinity for MyD88 and an increased affinity for the effector protein TRAF6. Phosphorylation of IRAK1 is therefore required for downstream signalling to NF-kB (Cao et al., 1996b).

IRAK1 is degraded following IL1 stimulation, and it is thought that this is signalled by IRAK1 phosphorylation. Inhibition of the proteasome has been shown to prevent degradation of phosphorylated IRAK1 and correspondingly increase the amount of IL-1 receptor that can be coprecipitated with IRAK1 (Yamin and Miller, 1997). It has been suggested that this IL1-dependent degradation of IRAK1 represents a negative feedback mechanism to terminate the IL1 response, and may be the mechanism whereby cells become desensitised after prolonged exposure to IL1.

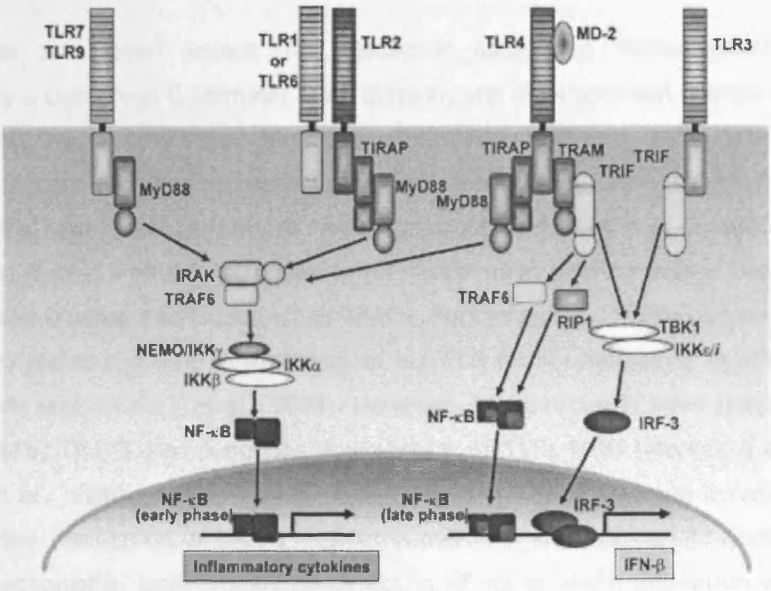

Figure 1.2. TLR signaling pathways

Intracellular signaling downstream of TLRs is initiated by the cytoplasmic TIR domain which binds to the following TIR domain-containing adaptor proteins.

MyD88 recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKß and NEMO/IKKγ. The IKK complex phosphorylates IκB, resulting in nuclear translocation of NF-κB which induces expression of inflammatory cytokines.

TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4.

TRIF is essential for the MyD88-independent pathway in which activation of IRF-3 and induction of IFN-ß are activated downstream of TLR3 and TLR4. Non-typical IKKs, IKK*i*/IKKε, and TBK1 mediate activation of IRF-3 downstream of TRIF.

A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

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1.2.2.6 TRAFs

Currently there are seven known TNF Receptor associated factor (TRAF) proteins, characterised by a conserved C terminal TRAF domain, and an N terminal domain containing a RING finger, and five or seven zinc-finger structures (Lee and Lee, 2002). The C terminal TRAF domain is required for self-association and interaction with receptors and other signaling proteins, and the N terminal is the effector domain. TRAF6 is unique among TRAF proteins: TRAFs1-3, and 5 bind to CD40 and RANK via common binding sites in the cytoplasmic tails, whereas the binding site is distinct in TRAF6. Furthermore, TRAF6, but not other TRAF proteins, is involved in signaling downstream of IL1/TLR family members, in addition to the TNFR superfamily (Kobayashi T et al., 2004). However, it has recently been suggested that in addition to TRAF6, TRAF3 also functions downstream of TLRs Both (Hacker H et al., 2006, Oganesyan G et al., 2006). Analysis of mice deficient for TRAF3 indicate a requirement for this protein in the production of type I interferon and IL10 via both MyD88-dependent and -independent mechanisms. Interestingly no defect in NF-κB or MAPK activation was observed (Hacker H et al., 2006).

It has been shown that inducing clustering of the effector domains of TRAF6 is sufficient to activate the proteins (Lee and Lee, 2002). This suggests that IRAK1 functions to activate TRAF6 via clustering its effector domain. The reduced affinity of IRAK1 for MyD88 means that IRAK1 and TRAF6 dissociate from the receptor complex and interact at the plasma membrane with a second heteromultimeric protein complex consisting of TGFβ-activated kinase1 (TAK1), and two TAK1 binding proteins, TAB1 and TAB2. TAK1 is a MAP3K protein originally identified in TGFβ-induced signal transduction (Yamaguchi et al., 1995).

IRAK1 then mediates translocation of this multiprotein assembly to the cytosol where further proteins necessary for activation of TAK1 kinase activity associate. IRAK1 itself does not translocate to the cytosol, but remains associated with the plasma membrane where it is degraded.

The E2 ligases Ubc13 and Uev1A are thought to mediate TAK1 activation. These proteins have been shown to catalyse the assembly of a polyubiquitin chain on TRAF6 (Deng et al., 2000), and this promotes TRAF6-mediated activation of TAK1 (Wang et al., 2001). Activated TAK1 then phosphorylates IKKs as well as MAP2Ks (see section 1.2.4)

Other TRAF6 binding proteins have been demonstrated, suggesting activation of multiple downstream signaling cascades. For example, TRAF6 has been shown to activate p62, which in turn activates PKCζ, and TRAF6 is also capable of activating ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), which is a MEKK1 activator. Although both PKCζ and MEKK1 have been suggested to activate IKK, no defects in NF-κB signaling were observed in the respective knockout mice, however this could reflect redundancy in IKK activatory proteins.

1.2.3 Limitations to studying intracellular signaling pathways in DC

The signal transduction events underlying the maturation process of DC have only recently started to undergo serious investigation. Such studies are limited by the fact that it is relatively difficult to obtain DC of high quality and quantity, since cells are largely obtained by differentiation from peripheral blood monocytes or bone marrow precursors. A number of monocyte-like cell lines exist, although their suitability as dendritic cell models is controversial. A good understanding of the role of different signaling pathways in DC is therefore limited by the use in different laboratories of a large selection of dendritic cell models, differentiated from different species and tissue types or cell line precursors.

To induce signal activation, different maturation stimuli are employed throughout the literature, at hugely variable concentrations, for example, in the case of LPS, concentrations as diverse as 1ng/ml and 10μg/ml are frequently employed. The source of such variation lies in the difficulty in obtaining accurate measurements of physiological conditions. To overcome this problem, cocktails of known DC maturation stimuli are sometimes used to stimulate the cells. Whilst being physiologically more realistic, such an approach complicates conclusions about the activation of specific signaling pathways.

Dendritic cells are notoriously difficult cells to manipulate, and the results of many biochemical approaches must be interpreted with care. For example, it has recently been shown that merely the process of transfecting DC is sufficient to activate them (Martin et al., 2006).

Most of what is known about intracellular signaling pathways in DC has been elucidated using two approaches, transgenic mice lacking specific genes, and studies with pharmacological inhibitors. Both of these approaches have potential limitations. Frequently, mice engineered to lack a single specific signaling protein fail to demonstrate significant phenotypic

alterations. This is almost certainly due to redundancy and hence reconstituted function between isoforms of target proteins, rather than a lack of function on the part of the protein *per se*. Given the ubiquitous nature of many of the signaling pathways under investigation, multiple knockout mice are frequently not viable, presumably due to roles of the signaling molecules in development. Clearly, mice models lacking multiple related genes in a dendritic cell-specific manner would be the optimum tools. However such models would be technically challenging to manufacture and have not yet been widely reported for signaling proteins.

By far the most commonly employed approach to analysing signaling pathways in dendritic cells is the use of pharmacological protein-specific inhibitors. This approach has the advantage of being cheap, easy, and quick. However, results of inhibitor studies should be interpreted with care. The first problem is that one can never truly be sure that the inhibitor does not interfere with additional unforeseen pathways. Manufacturers producing inhibitors usually assay for efficacy of the inhibitors using *in vitro* systems involving truncated proteins corresponding to just the catalytic domains. Clearly, the effect of the inhibitor on every possible protein cannot be examined. Also, unforeseen inhibitory mechanisms such as stearic effects rather than direct competitive inhibitory mechanisms cannot be fully assessed. The thalidomide tragedy, and the recent emergence of cerebrovascular disease in people treated with the cox2-inhibitory form of aspirin, are two amongst many other examples that testify to such unforeseen effects of inhibitors.

Furthermore, the specificity of a pharmacological reagent is a function of the concentration at which it is used - and at higher concentrations other signaling proteins will also be inhibited. Many studies use inhibitors vastly in excess of the concentrations at which they are specific for their target protein. For example the p38 inhibitor SB203580 has been suggested to have non-specific effects at concentrations as low as 3μM (Lali et al., 2000), although it's use at 50μM or even 100μM is frequently reported. Furthermore, this inhibitor directly inhibits the catalytic capacity of the p38 enzyme; it does not therefore prevent p38 phosphorylation by upstream activatory proteins (Tong et al., 1997). However, in many studies, efficacy of the SB203580 inhibitor is shown as a simple prevention of p38 phosphorylation - the authors are in fact indicating their own misuse of the inhibitor, and non-specific inhibition of upstream kinases!

Despite the misgivings outlined above, important features of different signaling pathways in DC can be elucidated by comparing observations obtained using different experimental approaches for common themes or functional traits associated with particular signaling

molecules. The following discussion attempts to clarify the most prominent themes of signaling pathways controlling dendritic cell maturation.

1.2.4 Mitogen-activated protein kinases

1.2.4.1 Introduction

Mitogen-activated protein kinase (MAPK) pathways are found in all eukaryotic cells. They are a multi-purpose signal transduction mechanism that can be harnessed to couple diverse stimuli to various effector systems. It is useful to consider that cells use MAPKs to prime themselves to produce a particular outcome in response to a given stimulus. This is achieved by using a MAPK pathway to couple the appropriate receptor to a particular set of transcription factors or other effector proteins. In this way different cell types can respond differently to the same stimulus, or indeed produce the same outcome to different stimulations.

Considered in the simplest terms, MAPK pathways are composed of a central three-tiered core-signaling module in which the sequential members are activated by dual phosphorylation. The core modules consist of the effector MAPKs, which are activated by upstream proteins, called MAPK kinases, or MAP2Ks. In turn, MAP2Ks are activated by MAP2K kinases, or MAP3Ks. MAPKs and MAP2Ks share a high degree of sequence and structural similarity, being composed of two lobes, representing the N- and C-terminal domains, joined by flexible hinge region at which the catalytic active site is found. The proteins are activated by dual phosphorylation on a disordered activatory region known as the T-loop. In MAPKs dual phosphorylation is upon Thr and Tyr residues in a conserved T X Y motif, whereas in MAP2Ks phosphorylation occurs on ser and ser/thr residues. The dual phosphorylation induces an ordering of the loop structure to stabilise the negative charges, which results in a realignment of the N- and C-terminal lobes and activation of the catalytic site. Clearly, dephosphorylation results in deactivation of the proteins via the reverse mechanism.

The number, diversity and enzymology of the ser/thr kinases that lie upstream of the MAP2Ks and act as MAP3Ks is immense. The enormous heterogeneity in this family of proteins is testament to the huge diversity in MAPK-activating stimuli. Generally, MAP3K activation is via membrane recruitment, oligomerization, and phosphorylation. One family of MAP3Ks, the mixed lineage kinases, will be considered in more detail in section 1.3. In contrast to the MAP3Ks, only three families of MAPKs have been identified in mammals, the extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK), and p38 kinase, and all three

MAPKs phosphorylate target substrates on serine or threonine residues followed by a proline; however, substrate selectivity is often conferred by specific interaction motifs located on physiological substrates.

ERK is mainly regulated by MAP3Ks of the Raf family via two MAP2Ks, MEK1 and MEK2. ERK exists in two alternatively spliced forms, of 42 and 44 KDa, and is characterised by an activatory Thr-Glu-Tyr activatory motif.

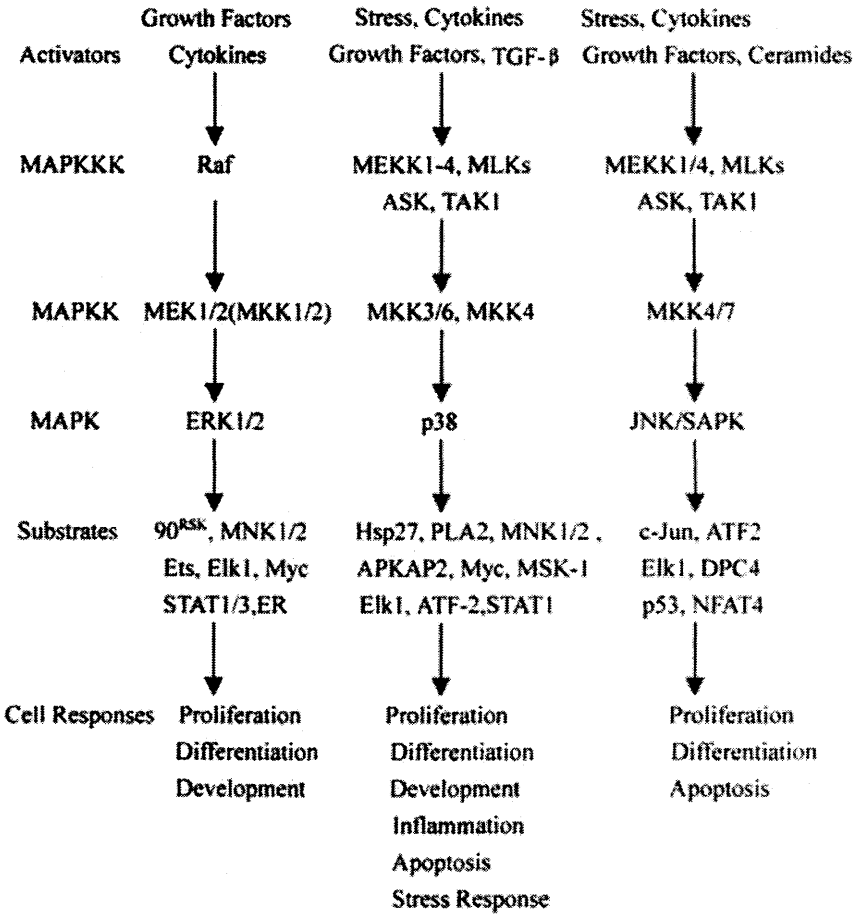

Figure 1.3. MAPK pathways

Major MAP kinase cascades in mammalian cells.

Zhang and Liu *Cell Research* **12**; 9-18 (2002)

JNK and p38 are often collectively referred to as stress-activated protein kinases, or SAPK, due to their important roles in stress and inflammatory responses. Three JNK isoforms exist, JNK1, JNK2, and JNK3, and all can be spliced into 46 and 54 KDa proteins, although the significance of the different isoforms is not clear. Four p38 isoforms exist, p38α-δ, all of 38KDa. The action of specific inhibitors known as cytokine-suppressive anti-inflammatory drugs (CSAIDs) facilitated the discovery of p38, the crystal structure of which has since been determined in complex with the most extensively characterised CSAID, SB203580 (Fitzgerald et al., 2003). JNK proteins possess a proline residue separating the Thr and Tyr residues of the phosphorylation motif, whereas p38 proteins are characterised by a glycine residue in this position. JNK is activated by MKK4 and MKK7 MAP2Ks, and p38 by MKK3 and MKK6 (Kyriakis and Avruch, 2001). The MAP3Ks upstream of these proteins are numerous, and are often promiscuous in their ability to target JNK and p38. Figure 1.3 outlines the organisation of MAPK pathways.

For reasons outlined in the section above, there are comparatively few studies into the roles of MAPKs in DC, and almost all such investigations have involved the use of specific inhibitors. However, JNK-specific inhibitors have only recently become available, and for this reason, the roles of p38 and ERK pathways in DC have been far more extensively studied. Investigations have demonstrated that activation of MAPK is important for regulation of survival, maturation, cytokine secretion, and morphology of DC, and I will attempt to review the results of these studies in the following sections.

1.2.4.2 Control of DC maturation

As discussed above, ligation of various DC surface receptors, including TLRs, CD40, and TNFR, is associated with the induction of DC maturation and is always accompanied by activation of p38 and ERK MAPKs to varying extents. Activation of JNK is not consistently observed, suggesting that this is a non-essential pathway in controlling DC maturation. Where it is observed, activation of the ERK, JNK, and p38 pathways occurs rapidly (within 15-30 minutes), is transient (absent by 4 hours), and with similar kinetics for each of the MAPKs (figure 4.4).

Interestingly, alterations in the level of stimulation affect the degree of MAPK phosphorylation observed, but never the kinetics of activation. Frequently, the threshold for activation of JNK is observed to occur at higher concentrations than that of p38 and ERK (Nakagawa et al., 2004; Bunyard et al., 2003). The idea of different signaling pathways activated at different concentrations is clearly an attractive one given that the level of

stimulation is one of the determinants that has been hypothesised to regulate the phenotype of the mature DC (Luft et al., 2004).

Adding further weight to the importance of MAPKs in control of DC maturation, several immunosuppressive drugs have been identified which mediate their effects via interference of these pathways. For example, epigallocatechin-3-gallate (EGCG) inhibits the maturation of murine DC induced by LPS, as measured by upregulation of surface markers CD80, CD86, and MHC molecules, and secretion of IL12 (Ahn et al., 2004). Biochemical analysis indicates that EGCG mediates these effects via inhibition of p38, JNK, and ERK activation in response to LPS, although the mechanism for this is unclear. Similarly, parthenolide (PTL) inhibits upregulation of LPS-, but not TNFα-induced CD80, CD83, CD86, CD40, and HLA-DR, on DC. PTL also prevents LPS-, but not TNFα-induced p38 and MKK3/6 phosphorylation (Uchi et al., 2002).

Clearly PTL mediates its effects in the LPS signaling pathway upstream of the point at which the LPS and TNFα signal pathways integrate. This observation is particularly interesting because it indicates that signaling pathways induced by different DC maturation stimuli, which operate though discrete surface receptors, converge to activate MAPKs, underlining the importance of these pathways in DC maturation. Indeed the idea of a central role for MAPK independent of the nature of the stimulus in the process of DC maturation is reinforced by numerous other examples. Lymphocyte activation gene 3 (LAG3) is expressed on activated T cells and ligates MHC class II molecules to induce DC maturation. Via the use of specific inhibitors, ERK and p38 activation was shown to be critical for TNFα secretion and upregulation of various surface markers in response to LAG3 stimulation (Andreae et al., 2003). Other ligands including CD40L (Luft et al., 2004; Vidalain et al., 2000), TNFα (Cruz et al., 1999; Sato et al., 1999), TLR ligands (Ardeshna et al., 2000; Re and Strominger, 2001), contact sensitizers (Arrighi et al., 2001; Boisleve et al., 2004), and nicotine (Aicher et al., 2003) have also been shown to induce DC maturation via a MAPK-dependent mechanism.

Current models suggest that by far the most important of the MAPKs in controlling DC maturation is p38. The upregulation of surface markers such as CD40, CD54, CD80, CD83, CD86, HLA-DR and secreted cytokines including IL6, IL8, IL10, IL12, and TNFα are inhibited by p38 inhibition, (Messmer et al., 2004; Pollara et al., 2004; Arrighi et al., 2001; Nakahara et al., 2004), and the degree of p38 phosphorylation has been shown to correlate with the level of expression of markers of DC maturation such as CD86 (Pollara et al., 2004).

Several of the physiological properties of DC that are altered upon maturation are also regulated by p38. Increased expression of α4 integrins, which mediate binding to the extracellular matrix protein fibronectin, and which are thought to reflect the DC migration process, are inhibited by p38, but not ERK inhibition (Puig-Kroger et al., 2000). CCR7 expression and DC migration is prevented by p38 inhibition (Ardeshna et al., 2002; Boisleve et al., 2004). *In vitro* T cell stimulatory capacity is sensitive to p38 inhibition (Arrighi et al., 2001; Nakahara et al., 2004; Yu et al., 2004) (although the results of these studies are difficult to interpret since it is impossible to exclude effects of diffusion of the inhibitor out of the DC and into the T cells). The downregulation of endocytic capacity observed upon maturation has also been suggested to be mediated by p38 (Arrighi et al., 2001; Nakahara et al., 2004), although several studies refute this (Ardeshna et al., 2000; Puig-Kroger et al., 2001), and indeed the regulation of DC endocytosis remains controversial.

Many of these functions of p38 have been deduced using the SB203580 inhibitor. A recent study using an SB203580-resistant p38α protein has confirmed the involvement of this isoform in the LPS-induced expression of IL6, IL10, and TNFα (Guo et al., 2003a).

In one of the few non-pharmacological-based investigations into the role of p38 in DC, germline MKK3-deficient mice were generated (Lu et al., 1999). Macrophages derived from the mice were found to be defective in p38 activation in response to some stimuli such as LPS, but not others, such as sorbitol. Given that p38 is activated by MKK3 and MKK6, this provides a useful insight into the relative contribution of the two MAP2Ks. Reconstitution with MKK3 restored LPS-induced p38 activation, suggesting that MKK3 is the most important MAP2K in activating p38 in response to TLR4 stimulation. In MKK3-deficient DC, LPS- and CD40L-induced IL12 production was significantly inhibited, suggesting that p38 is important for IL12 production.

Despite being significantly diminished by p38 inhibition (Arrighi et al., 2001), secretion of TNFα and IL6 were not reduced in the MKK3 knockout mouse suggesting that the regulation of these two cytokines is MKK6-dependent although the mechanism underlying the MAP2K preferences of different DC maturation markers is unclear (Lu et al., 1999).

Interestingly, two p38 MAPK isoforms have been identified in *Drosophila* that appear to regulate anti-microbial gene expression (Han et al., 1998). These D-p38 proteins are also sensitive to inhibition by the SB203580 p38 inhibitor. As in mammalian cells, LPS was shown to activate D-p38 MAPK. However, in contrast to the role of p38 in mammals, activation of D-p38

was associated with a suppression of immune gene transcription, as demonstrated by overexpression of D-p38, and enhanced LPS-induced expression of anti-microbial genes in the presence of SB203580. Nonetheless, these studies illustrate the importance of p38 in the control of immune responses in higher organisms.

Interestingly, D-JNK has also been identified, and shown to be activated during the immune response in *Drosophila*. In mammalian systems the role of JNK in controlling DC maturation is not well understood. However, it has recently been demonstrated that JNK inhibition does not prevent induction of T cell proliferation, cytokine production or endocytosis, although there is a small reduction in the level of expression of CD54 and CD80 in response to LPS (Nakahara et al., 2004), suggesting that JNK plays only a minor role in the control of DC maturation.

Several studies indicate that the ERK pathway has a negative regulatory role in controlling DC maturation (Puig-Kroger et al., 2001), while other studies indicate that ERK has no apparent role (Ardeshna et al., 2000). This discrepancy appears to result from the utilisation of different levels of stimulation. For example, with low stimulation, such as 10ng/ml LPS or using TNFα or CD40L, ERK negatively regulates the expression of surface markers, including CD83, CD49d, CD86, and HLA-DR (Arrighi et al., 2001; Puig-Kroger et al., 2001; Aicher et al., 1999). However, when 100ng/ml or more of LPS is used to stimulate the DC, ERK inhibition has no effect on the maturation program (Kikuchi et al., 2003; Puig-Kroger et al., 2000). Rather than being a straightforward negative regulator, it is now thought that the role of the ERK pathway in modulating DC maturation is in fact more complex than this, and regulates the effector T cell phenotype of the resulting immune response.

1.2.4.3 Effector T cell polarization

In all published studies of MAPK signaling induced by CD40L in DC, inhibition of p38 prevents DC maturation in terms of migration (Luft et al., 2004), IFNγ (Yu et al., 2004), and IL12 secretion (Vidalain et al., 2000; Kikuchi et al., 2003; Aicher et al., 1999), upregulation of surface molecules CD80, CD83, CD86, HLA-DR, and allostimulatory capacity (Yu et al., 2004). The effects of JNK inhibition on CD40L-induced DC maturation have not been reported, possibly reflecting an absence of phenotype. CD40L-induced ERK phosphorylation occurs with identical kinetics to that of p38. ERK inhibition has no effect on the upregulation of surface markers (Yu et al., 2004), but does result in increased IL12 production and DC migration in response to CD40L (Luft et al., 2004). This is interesting because it not only suggests that different aspects of DC maturation are regulated independently, but it also implicates the

ERK pathway in a negative-regulatory capacity in some facets of this maturation program. According to this hypothesis, relative to DC in which ERK is activated, absence of ERK activation would induce DC with mature surface phenotype, but greater capacity to secrete IL12. Such DC would be archetypal Th1-inducers, and thus the function of the ERK pathway in response to CD40L may be to suppress the Th1-like response.

The capacity of ERK to suppress Th1 responses appears to be a general theme regulating the phenotype of the mature DC in response to all DC maturation stimuli. ERK2 knockout mice are not viable. ERK1 knockouts however, demonstrate significantly enhanced production of IL12p70 in response to LPS, whereas the induction of IL10 is significantly reduced in these mice. Secretion of other cytokines, such as IL6, is unaffected (Dillon et al., 2004). Accordingly, a 5-fold increase in IL12 production in response to TNFα is observed following preincubation with a pharmacological ERK inhibitor (Puig-Kroger et al., 2001), and this is mirrored in response to LPS (Dillon et al., 2004; Xia and Kao, 2003), and the ability to synthesise IL10 is significantly retarded (Dillon et al., 2004; Xia and Kao, 2003). DC derived from mice engineered to lack the ERK-dependent transcription factor c-fos respond in an identical fashion to ERK1-deficient DC, and demonstrate an enhanced Th1 phenotype, suggesting that the suppression of Th1 responses by ERK occurs via a c-fos dependent mechanism.

Despite these findings on ERK- or c-fos-deficient mice, variable effects of ERK inhibition on the level of IL12 secretion have been reported, ranging from no effect (Kang et al., 2004; Nakahara et al., 2004; Dillon et al., 2004; Vidalain et al., 2000) to a strong enhancement (Aiba et al., 2003; Kikuchi et al., 2003; Puig-Kroger et al., 2001). This is may reflect the utilisation of different stimuli at different concentrations. There is no argument, however, that ERK is required for IL10 synthesis, and it is therefore possible that the reported increases in IL12 production upon ERK inhibition are actually mediated indirectly via reduced IL10 secretion. Indeed, addition of exogenous IL10 can reverse the upregulation of IL12 observed following ERK inhibition (Xia and Kao, 2003). Regardless of the exact mechanism, the observations that p38 controls IL12 production, and ERK is not required for IL12, but is essential for IL10 synthesis suggest that the T cell profile of the resulting immune response is largely controlled by the relative activities of these two MAPKs. Interestingly, many investigations report that inhibiting either of the p38 or ERK MAPKs results in enhanced phosphorylation of the other upon stimulation (Wilflingseder et al., 2004; Valentinis et al., 2005; Puig-Kroger et al., 2001). These experiments are performed with an incubation period of only 60 minutes, ruling out a cytokine feedback system in this case. A possible mechanism

for this antagonism between the p38 and ERK MAPK pathways is via the regulation of phosphatase activity. Indeed, phosphorylation and stabilisation of a JNK phosphatase, MKP7, by ERK has recently been demonstrated, suggesting a mechanism for ERK-induced JNK inactivation (Masuda et al., 2003; Katagiri et al., 2005), and a similar interaction may mediate regulation of ERK by p38 and vice-versa.

IL12 treatment of DC stimulates p38 phosphorylation (Nagayama et al., 2000), and IL10 induces ERK activation (Mathur et al., 2004). This observation, in combination with the proposed ERK-p38 antagonism outlined above, suggests that IL12 and IL10 present in the extracellular milieu will enhance p38 or ERK signaling respectively within the DC, thus stimulating their own production and therefore reinforcing the prevailing T cell polarization. A similar mechanism has been proposed to operate in macrophages in response to CD40 (Mathur et al., 2004), and CpG DNA stimulation (Yi et al., 2002; Hacker et al., 1999). In support of this model is the observation that MKK3 knockout mice demonstrate impaired Th1 responses (Lu et al., 1999).

To evade immune detection, many organisms are known to skew the effector T cell profile of an immune response. It has been suggested that *Leishmania* amastigotes, for example, exploit this mechanism of MAPK regulation to inhibit a Th1 outcome by enhancing ERK activation following CD40 stimulation (Mathur et al., 2004).

It was outlined above that the outcome of DC maturation is controlled by the integration of multiple signals from pathogens, inflammatory mediators, chemokines, cell-cell contact, etc. Many of these ligands will induce MAPK signaling and clearly the balance of p38 and ERK that is induced will be critical in the determination of the Th1/Th2/Treg bias of the mature DC.

One of the major factors implicated in the control of the effector T cell profile is the identity of the DC maturation stimulus. Recently several studies have been published comparing the ability of different TLR ligands to induce Th1 and Th2 responses. In particular, TLR2 ligands such as Pam3cys and schistosome egg antigen (SEA) have been shown to direct the T helper cell phenotype towards the Th2 pathway, and in agreement with the above model, this has been shown to occur via a mechanism dependent on the ERK pathway and the c-fos transcription factor (Dillon et al., 2004; Agrawal et al., 2003; Kane et al., 2004). In particular, via ERK activation and c-fos stabilisation, these two stimuli induced upregulation of surface markers in the absence of IL12p70 secretion, and induced T cell proliferation with subsequent IL4 and IL5 production. ERK inhibition, or use of ERK1- or c-fos-deficient DC is

sufficient to increase IL12p70 and decrease IL10 production in response to these TLR2 ligands to levels comparable to those induced by LPS or flagellin (Dillon et al., 2004).

In view of these studies, it would seem reasonable to assume that ligation of different TLRs activates DC to induce Th1 or Th2 responses by the preferential activation of p38 or ERK, perhaps via different intracellular adaptor proteins. However LNFPIII is a helminth carbohydrate ligand of TLR4, which induces DC to stimulate high IL4 and low IFNγ from responding T cells, and thus can be considered a Th2 ligand. LNFPIII induces strong ERK and little p38 phosphorylation (Thomas et al., 2003), whereas LPS induces the reciprocal pattern of MAPK activation via the same receptor. How is this achieved? Currently no structural studies of any TLRs have been reported, but one possible mechanism is that LNFPIII utilises a second, additional receptor, which favours ERK over p38 activation. Although TLR4-deficient mice do not respond to LNFPIII, an additional receptor may still exist which is incapable of inducing maturation *per se*, but can bias the outcome of TLR4-induced maturation. An alternative possibility is that strength of the signal is somehow capable of determining the polarization of the responding T cells.

Signal strength has previously been postulated to determine the Th1/Th2 bias, with strong, persistent stimulation associated with a Th1-type response, and transient, weaker stimulation, inducing Th2 (Boonstra et al., 2003a). In support of this model, using modified CD40L molecules that induced strong or weak signaling, Luft *et al* recently showed that strong and sustained signaling was associated with IL12p70 production (Luft et al., 2004). With reference to the above example, if LNFPIII has a lower affinity for TLR4 than does LPS, then the DC would translate this into a Th2 outcome. Indeed, it was recently reported that LNFPIII induces a rapid, but transient NF-κB activation, whereas LPS induces more persistent activation (Thomas et al., 2005). Furthermore, low dose LPS has previously been demonstrated to promote Th2 development (Eisenbarth et al., 2002). The precise mechanism for signal strength-mediated differential intracellular signaling is unclear, however.

1.2.4.4 Mechanisms of MAPK action in DC

In MKK3-deficient DC, as discussed above, LPS- and CD40L-induced IL12 production was significantly inhibited and this was found to occur at the level of IL12p40 transcription. The IL12p40 promoter has been well characterised and is controlled by several transcription factors including C/EBP, and ets-2-related factor, which are both phosphorylated and activated by p38 (Williamson et al., 2005; Yordy and Muise-Helmericks, 2000). This highlights

the role of p38 in the control of cytokine production via the regulation of transcription factors.

In addition to directly regulating the activity of transcription factors, p38 can also control gene expression in DC by modulating higher order chromatin structure. Before transcriptional regulators can access their sequence-specific binding sites, a transition from a condensed to decondensed chromatin structure takes place. The activation of transcription is then accompanied by the remodelling of specific nucleosomes, and this is controlled in part by phosphorylation on the constituent histone proteins. Addition of electronegative phosphate groups results in a reduced affinity for DNA, mediated by the repulsive forces between the phosphate groups and the negatively charged backbones of the nucleic acid double helix. It is proposed that in response to appropriate stimulation, p38 induces phosphorylation of a serine residue (ser10) in histone H3. This sensitises the histone to hyperacetylation on neighbouring lysine residues, further increasing the local electronegativity and permitting transcriptional activation (Saccani et al., 2002). Serine to alanine mutation of this residue abrogates transcription of specific inducible genes underlining the importance of this mechanism. The serine of H3 is not followed by a proline, and is therefore not a direct p38 target. Several candidate proteins, however, may function as intermediates such as the mitogen and stress activated protein kinase-1 (MSK1), which is a p38 target protein and known to phosphorylate histone H3 on this serine residue (Thomson et al., 1999a).

MSK1 is also an ERK target, raising the possibility that the ERK and p38 pathways cooperate in the induction of histone H3 phosphorylation by this protein. However, other known histone H3 kinases also exist, with differing MAPK dependency, such as the ERK-specific ribosomal S6 kinase-2 (RSK2) (Thomson et al., 1999b). This suggests that different stimuli could induce overlapping subsets of genes via different H3 kinases, depending upon the relative levels of ERK and p38 activation.

Following histone H3 phosphorylation, the transcription factor NF-κB is recruited to a subset of genes that are rapidly induced in response to microbial products, and regulated by the p38 pathway (Saccani et al., 2002). The model therefore describes a system by which the p38 and NF-κB pathways, which are both known to be critical for DC maturation, cooperate via p38-dependent enhanced accessibility of NF-κB binding sites. Presumably, the enhanced promoter accessibility also facilitates binding of other transcription factors, such as those that are ERK or JNK dependent, further reinforcing the cross-talk between signaling pathways.

Another mechanism by which p38 is known to control production of inflammatory proteins is via mRNA stabilisation. Many mRNAs contain in their 3' untranslated regions an AU-rich element (ARE), which sensitises the transcript to degradation but also permits p38-dependent stabilisation. The mechanism for this stabilisation is not known (Dean et al., 2004). Interestingly, the anti-inflammatory TGFß protein has been shown to selectively inhibit LPS-induced chemokine mRNA stabilisation (Dai et al., 2003).

MAPKs also regulate function at the protein level. For example, ERK activation in DC mediates IL1 secretion via activation of caspase 1, which is required for cleavage of pro-IL1 yielding the active IL1 molecule (Guo et al., 2003b).

The direct and indirect roles of p38 in gene modulation probably operate in parallel. Some genes, for example, such as IL8 and MCP1, are p38-dependent and associated with H3 phosphorylation. Others, including MIP1α and TNFα, are not associated with H3 phosphorylation. Since studies with the p38 inhibitor have shown that TNFα secretion is p38 dependent, p38 must regulate different genes at different levels.

1.2.2.5 Survival

In addition to their role in regulating DC maturation, there is evidence to suggest that MAPKs function in the control of DC survival. The duration of stimulation of naïve T cells is determined by the life span of the activatory DC - indeed it has been postulated that CD40 and MHC class II transmit antagonistic survival signals from T cells to DC (McLellan et al., 2000). The control of DC survival and the induction of apoptosis is therefore a critical factor regulating specific immunity. All three MAPKs have been implicated in this process, although in contrast to the control of maturation, there are very few studies into the role of MAPKs in DC survival.

The p38 pathway appears to possess a pro-apoptotic capacity. The specific inhibitor SB203580 inhibits caspase 3 activation and thus apoptosis of DC in response to the immunosuppressive drug triptolide (Liu et al., 2004). SB203580 also inhibits UV-induced DC apoptosis (Nakagawa et al., 2004).

The role of the JNK pathway in DC survival has not been well studied. However a pro-apoptotic capacity for JNK is well established in other cell types (Bharti and Aggarwal, 2004), and such a role in DC is suggested by the observation that in response to ligation of the receptor-activator of NF-κB (RANK) in raw cells, JNK is required for apoptosis (Bharti et al.,

2004). The role of p38 and JNK in DC maturation and survival is explored in more detail in chapter 3 of this thesis.

Several studies implicate the ERK pathway in a pro-survival capacity (Rescigno et al., 1998). Human Osteoclast-Associated Receptor (hOSCAR) is Fc Receptor-associated and signals via the intracellular ITAM of this affiliated molecule. Ligation of hOSCAR results in expression of anti-apoptotic proteins such as BCL_{XL}, and this was prevented by ERK inhibition (Merck et al., 2005). Furthermore, LPS has been proposed to induce DC survival by activating the ERK pathway via a family of caspase-like proteins (Franchi et al., 2003). The pro-survival role of ERK is controversial, however, since several studies find no alteration in the level of DC survival upon ERK inhibition (Guo et al., 2003b; Park et al., 2002).

It is interesting that the p38 and ERK pathways apparently play opposing roles in survival, reminiscent of their roles in determining the T cell polarization. Thus, a model of the overall picture of MAPKs in DC emerges. High levels of stimulation induce a Th1-like outcome via strong p38 activation and this is associated with apoptosis, perhaps reflecting a shortened window for T cell activation. On the other hand, weaker stimulation results in ERK activation and induction of Th2 responses, which are prolonged due to the pro-survival effect of the ERK pathway.

The MAPK pathways are obviously not the only signaling modules involved in regulating DC. The role of the PI3K and NF-κB pathways are reviewed below.

1.2.5 PI3K

Phosphatidylinositide-3'-OH kinases (PI3Ks) are a family of lipid kinases which catalyse the addition of a phosphate molecule specifically to the 3' position of the inositol ring of phosphoinositides (Toker and Cantley, 1997). Pleckstrin homology (PH) domain-containing proteins are recruited by these second messengers to the plasma membrane, and are then activated by phosphorylation before phosphorylating numerous protein targets.

Several studies have proposed a pro-survival role for PI3K in DC, consistent with observations in other cell types (Datta et al., 1999). In particular, studies with the PI3K-specific inhibitor LY294002 demonstrate increased DC apoptosis in response to ligation of hOSCAR (Merck et al., 2005), CD40 (Yu et al., 2004), TLR9 (Park et al., 2002), and TLR4 (Ardeshna et al., 2000). The mechanism for this is probably via the downstream PI3K effector protein, Akt1, which is known to phosphorylate and regulate a variety of pro-apoptotic regulators and also positively

regulates proteins that promote survival. CCR7 induces Akt1-dependent survival via sequential activation of Gi proteins and PI3K (Sanchez-Sanchez et al., 2004). Despite being activated by CCR7 ligation, PI3K is not involved in the control of chemotaxis or migratory speed (Riol-Blanco et al., 2005).

The role of PI3K in the regulation of DC maturation remains unclear. PI3K and Akt are activated by ligation of TLRs and members of the TNF family that signal via TRAF6 such as TLR4 (Termeer et al., 2002) and RANK (Bharti et al., 2004), suggesting a role in regulation of maturation (Yu et al., 2004; Ardeshna et al., 2000). However PI3K inhibition does not prevent maturation induced by TLR4 ligation (Termeer et al., 2002). PI3K has been shown to play an important role in IL12 secretion, although this function is not consistent between mouse and man. PI3K is required for IL12 secretion in humans (Re and Strominger, 2001; Yu et al., 2004). However, a knockout mouse engineered to lack PI3K showed increased IL12 production, and this negative regulatory effect of PI3K was also observed when mouse BMDC were treated with LY294002 (Fukao et al., 2002). The authors propose that the mechanism of PI3K-mediated IL12 suppression in murine DC is via inhibition of p38 by PI3K, perhaps at the level of MAP3Ks (Fukao et al., 2002). This discrepancy in IL12 control could reflect species differences between human and mouse, or perhaps may relate to the DC models used in the two systems.

1.2.6 NF-κB

The NF-κB family of transcription factors contains 5 members called Rel proteins: p65 (Rel A), p50/p105 (NF-κB1), c-Rel, p52/p100 (NF-κB2), and RelB. Functional NF-κB molecules consist of homo- or hetero-dimeric complexes of Rel proteins. Rel proteins are characterised by an N-terminal Rel-homology domain (RHD), which mediates DNA binding, nuclear localisation, and subunit dimerisation. Prior to stimulation, NF-κB proteins are sequestered in the cytoplasm by inhibitory proteins called IκBs (inhibitors of NF-κB). Upon stimulation, IκBs are phosphorylated, targeting them for ubiquitination and ultimately proteolytic degradation, releasing NF-κB to translocate into the nucleus and modulate gene expression (figure 1.4). The transcriptional activity of some NF-κB dimers is additionally regulated by phosphorylation.

There are several IκB proteins, although the pathway leading to the degradation of IκBα has been characterised in the greatest detail, and is known as the 'canonical' NF-κB pathway. As shown in figure 1.4, IκBα is phosphorylated by an IκB kinase complex (or 'signalosome'), comprising 2 kinase subunits IKK1 (or α), and IKK2 (or β), and a structural subunit NEMO. IKK1

and IKK2 can each phosphorylate IκBα on two serine residues, ser^{32} and ser^{36}, leading to the release of the NFκB heterodimer. Activation of the IKK complex by various agonists including TNFα and IL1 requires the MAP3Ks MEKK3 and TAK1, which are thought to phosphorylate the IKKs, thus providing a link between the MAPK and NF-κB pathways.

There is much evidence that NF-κB positively regulates DC maturation. Many DC maturation stimuli have been shown to induce NF-κB activation (Kang et al., 2004; Hegde et al., 2004; Ardeshna et al., 2000; Basu et al., 2000; Banki et al., 2003), and overexpression of IκBα in DC prevents the upregulation of CD40, CD80, CD83, CD86, and HLA-DR, and expression of cytokines including IL12, IL6, and TNFα (Yoshimura et al., 2001). Nuclear translocation of NF-κB subunits is observed following stimulation with DC maturation stimuli (Rescigno et al., 1998; Kim et al., 2004), and DC Maturation is preceded by an increase in DNA binding capacity of RelB, p50, p52, and c-Rel (Verhasselt et al., 1999; Neumann et al., 2000).

Several NF-κB inhibitors have been described and inhibit DC maturation: TLR2- and TLR4-induced upregulation of CD86 and HLA-DR is inhibited by pyrrolidine dithiocarbamate (PDTC) (Kim et al., 2004); SN50 inhibition of NF-kB results in partial inhibition of CD80, CD83, CD86, and HLA-DR in response to LPS (Ardeshna et al., 2000); TNFα secretion in response to LPS is inhibited by the NF-kB inhibitor CAPE (caffeic acid phenethyl ester) (Termeer et al., 2002); TPCK (N-alpha-Tosyl-L-phenylalanine chloromethyl ketone), prevents the partial upregulation of CD83 and CD86 induced by Polymyxin B (Valentinis et al., 2005).

NF-κB subunits are differentially recruited by various DC maturation stimuli (Hofer et al., 2001; Neumann et al., 2000). The role of individual NF-κB dimers has been addressed in the murine system using mice deficient for particular Rel proteins, and these studies indicate that a large degree of redundancy exists between the family members. Mice deficient for c-Rel showed no deficiency in IL12 production (Mason et al., 2002). Mice lacking both c-Rel and p50 proteins demonstrated impaired IL12 production, despite DC from these mice expressing normal levels of MHC and costimulatory molecules (Ouaaz et al., 2002). In agreement with these studies, RNAi inhibition of p50 in DC resulted in reduced IL12 secretion, while expression of HLA-DR and costimulatory molecules were not affected. These DC also induced T cells, which secreted less IFNγ revealing a requirement for p50 in DC for induction of Th1 immune responses. (Laderach et al., 2003). Furthermore, p50-deficient mice demonstrate defective IL4, IL5, and IL13 production and induce increased INFγ in response to the Th2 stimulus SEA (Artis et al., 2005). This was accompanied by defective OVA-specific $CD4^+$ T cell differentiation, IL4 and IL5 production. Interestingly, there is reduced ERK activation in the

p50-deficient DC, which is consistent with an abrogated Th2 response, however the mechanism for this ERK deficiency is not clear (Artis et al., 2005). Consistent with this observation is the finding that the ERK signal transduction pathway negatively regulates NF-κB DNA binding during TNFα-induced DC maturation (Puig-Kroger et al., 2001).

NF-κB has also been implicated in regulating DC Survival. Importantly, ligation of Fas on the surface of DC by Fas ligand during DC-T cell interaction induces pro-survival NF-κB activity that prevents the DC from entering apoptosis (Rescigno et al., 2000; Guo et al., 2003b). Additionally, CCR7 ligands CCL19 and CCL21 induce IkB degradation and NF-kB nuclear translocation, and NF-kB inhibitors NAC and SN50 prevent the pro-survival effects of these chemokines (Sanchez-Sanchez et al., 2004). Ligation of another DC receptor, RANK also has a pro-survival phenotype, and this is thought to be mediated via transcription of anti-apoptotic genes such as BCL_{XL} via NF-kB (Bharti et al., 2004). Furthermore, the NF-κB inhibitor PDTC makes DC susceptible to apoptosis, confirming that NF-kB-transcribed genes are involved in anti-apoptotic responses (Guo et al., 2003b).

1.2.7 Other signaling pathways

Given the ability of DC to integrate multiple ligand-induced signals derived from pathogens, inflammatory mediators, chemokines, cell-cell contact, etc, and translate them into a particular mature phenotype, these cells are likely to utilise many different families of signaling proteins. While the MAPK and NF-κB pathways have been shown to play major roles in controlling the outcome of the DC maturation process, the roles of many signaling pathways in this process await detailed investigation.

The PKC pathway is a good example of a family of signaling proteins that may be involved in the control of DC maturation, but have not been thoroughly investigated. LPS stimulation of DC results in PKC phosphorylation, and inhibition of PKC activation using bisindolylmaleimide, a pan-PKC inhibitor, prevents IL-12 production, but does not affect up-regulation of MHC class II, CD80 or CD86 (Aksoy et al., 2002). An identical outcome of inhibition of IL12 secretion but not surface marker expression was obtained in response to LPS in the presence of the PKA-specific inhibitor H89 (Galgani et al., 2004). In both studies IL12 inhibition was accompanied by activation of IL4-secreting T cells, suggesting that PKC and PKA are not involved in controlling maturation *per se*, but are involved in determining the resulting T cell profile. PKC has also been suggested to modulate the pro-apoptotic response of DC to MHC class II ligation (Bertho et al., 2002).

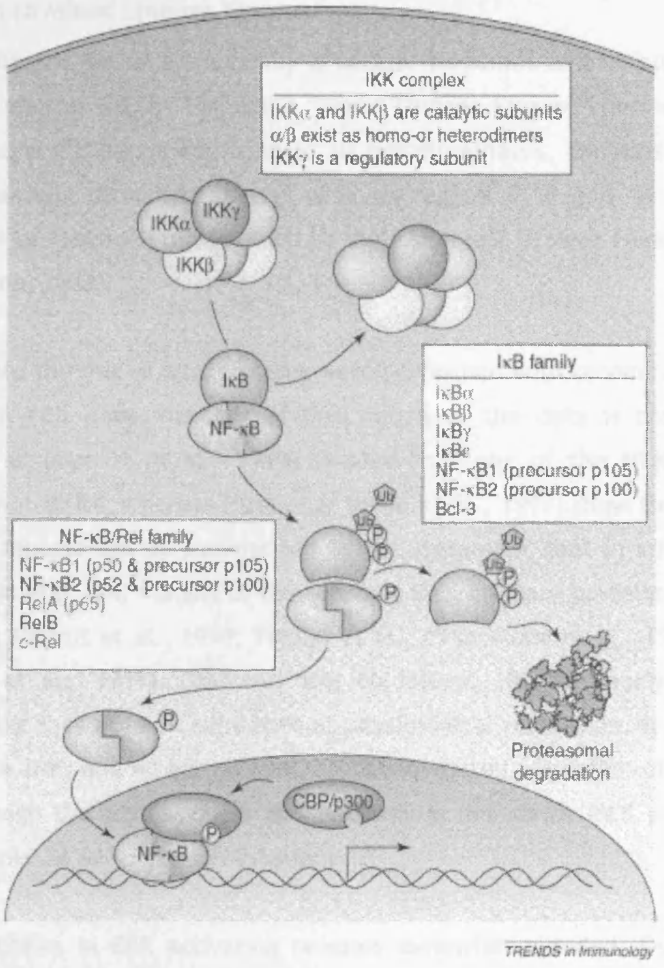

Figure 1.4. Model of NFκB activation

Canonical pathway of NFκB activation by IκB phosphorylation, ubiquitination, and degradation.

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1.3 Mixed Lineage Kinases

1.3.1 Introduction to Mixed Lineage Kinases

The Mixed Lineage Kinases (MLKs) are a family of serine/ threonine kinases all of which act as MAP3Ks (Gallo and Johnson, 2002). The name "Mixed Lineage Kinases" derives from the fact that of the 11 conserved subdomains found in all protein kinases, domains 1-8 of the MLK proteins resemble serine/ threonine kinases whereas regions 9,10 and 11 share sequence similarity with those of tyrosine kinases, such as the Fibroblast Growth Factor Receptor and Src (Gallo and Johnson, 2002).

Early experiments into the role of MLK proteins were performed with recombinant proteins or by overexpression in cell lines. Because of this, much of the data is contradictory. For example Merritt et al (Merritt et al., 1999) showed that one of the MLK proteins, DLK, activates MKK7 and not MKK4, whereas Hirai et al (Hirai et al., 1997) show that DLK activates MKK4. Six different reports can be summarised by the statement that *in vitro* MLK proteins can activate both p38 and JNK, via any of the relevant intermediary proteins, MKKs 3,4,6, or 7 (Hirai et al., 1997; Merritt et al., 1999; Tibbles et al., 1996; Rana et al., 1996; Cuenda and Dorow, 1998; Hirai et al., 1998). Obviously this statement, like the early experiments it describes, fails to take into account subtleties of physiological regulation. It is likely that all MLK proteins activate JNK, and all act via MKK7. MKK4-mediated activation of JNK occurs to a lesser degree, although the regulation of this pathway is not clear. MLK proteins can also activate p38 and this is via MKK3 phosphorylation.

The role of MLK proteins in ERK activation remains somewhat clouded. Overexpression of MLK3 has been shown to induce moderate activation of the MEK1-ERK protein kinase pathway. This activation was prevented by the MEK-specific inhibitor PD98059 or by expression of a kinase-deficient MEK1 mutant (Hartkamp et al., 1999). In contrast to this however, Shen *et al* demonstrate that although MLK3 can phosphorylate and activate MEK-1 directly *in vitro* and can induce MEK phosphorylation in COS-7 cells, this induction of MEK phosphorylation does not result in ERK activation *in vivo* (Shen et al., 2003). However a recent study reported that silencing of *mlk3* by RNAi suppressed mitogen and cytokine activation not only of JNK and p38, but of ERK as well (Chadee and Kyriakis, 2004b).

It therefore appears as though the picture of mixed lineage kinases as comparatively selective regulators of the JNK group of MAPKs is no longer valid. By discussing current theories regarding the regulation of activation and specificity of MLK proteins I will attempt to expand

and clarify this issue in the following literature review. It should be noted that MLK proteins have not previously been studied in DC, and the role of these proteins in the regulation of DC survival, maturation and morphology is central to the investigations presented in this thesis.

1.3.2 Types of MLK

It is believed that there are eight mammalian MLKs, categorised into three subfamilies on the basis of domain organisation and sequence similarity (figure 1.5):
1) MLKs; MLK1-4 (Dorow et al., 1993; Ing et al., 1994)
2) DLKs (Dual Leucine Zipper-Bearing Kinases); DLK and LZK (Holzman et al., 1994; Sakuma et al., 1997)
3) ZAKs (Zipper Sterile-α-Motif Kinases); ZAKα and ZAKβ (Liu et al., 2000; Gotoh et al., 2001)

MLK2, MLK3, DLK and LZK (Dorow et al., 1993; Ing et al., 1994; Sakuma et al., 1997) are the only MLKs whose biochemistry has been studied in any detail. This is probably because they are either widely expressed (MLK3, LZK), or are brain restricted (MLK2, DLK), and hence offer potential therapeutic targets for neurodegenerative diseases such as Parkinson's.

All eight MLKs contain a leucine zipper domain. These consist of one or two α-helices with predominantly non-aromatic lipophilic amino acids occupying one face, which facilitates protein multimerisation via hydrophobic interactions.

MLK1-4 share a high degree of homology in the N-terminal 500 or so amino acids, which incorporate several functional domains: an SH3, a kinase catalytic domain, the leucine zipper, a proline-rich region, and the CRIB domain which mediates binding to GTP-bound cdc42 and rac. In contrast to the amino terminal portion of the proteins, the carboxyl termini share little homology and vary greatly in size, possibly to mediate specificity and orchestrate interactions with various other proteins.

The DLK subfamily is identified by possession of two leucine zippers separated by a 31 amino acid spacer region. DLK and LZK share a high degree of homology.

The most recently identified MLK subfamily have been termed ZAKs due to the fact that the first identified member of the family, ZAKα, contains a sterile-α-motif (SAM): an independent globular domain of about 70 amino acids found in many signal transduction proteins. Biochemical and structural studies suggest that SAM domains mediate protein dimerisation. The second ZAK protein, ZAKβ, has a much smaller C-terminal domain than ZAKα and as a result lacks the SAM. The ZAK proteins are only loosely related to the other six MLKs, sharing

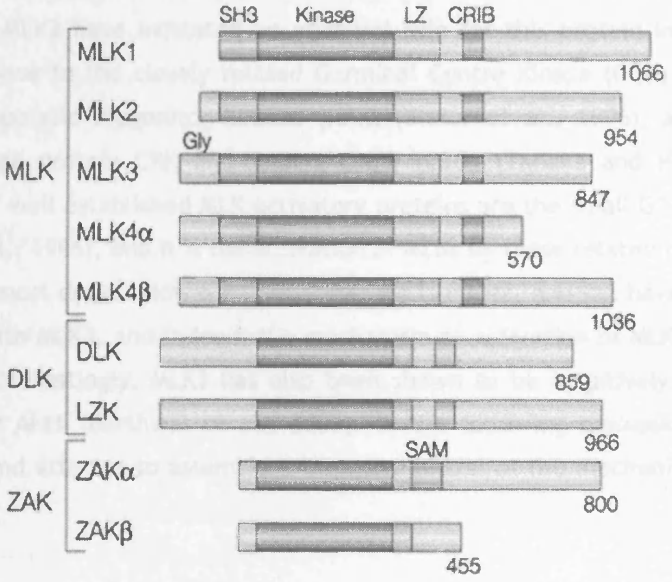

Figure 1.5. Mixed Lineage Kinases

Structures of mixed-lineage kinases (MLKs).

Domains: SH3 (Src-homology-3), kinase, LZ (leucine-zipper), CRIB (Cdc42/Rac-interactive binding domain), and SAM (sterile-α motif).

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sequence homology in the kinase catalytic region and bearing a small leucine zipper domain (Gotoh et al., 2001).

1.3.3 Activation Of MLKs

A diverse collection of MLK activatory proteins has been identified. Studies using dominant negative forms of MLK3 have indicated an essential role for this protein in mediating JNK activation in response to the closely related Germinal Centre Kinase (GCK) (Tibbles et al., 1996) and Hematopoietic Progenitor kinases (HPK) (Kiefer et al., 1996), and the guanine nucleotide exchange protein CRK SH3-binding GNFP (C3G) (Tanaka and Hanafusa, 1998). However, the most well established MLK activatory proteins are the small GTPases cdc42 and rac (Teramoto et al., 1996), and it is the activation of MLKs by these related proteins that has been examined in most detail. However, only HPK, Akt1, cdc42, and rac have been shown to directly interact with MLK3, and indeed, the mechanism of activation of MLK family proteins remains unclear. Interestingly, MLK3 has also been shown to be negatively regulated upon phosphorylation by Akt1 (Barthwal et al., 2003). In the following discussion I will review current evidence and attempt to assemble a tentative model of the mechanism of activation MLK proteins.

Phosphorylation is an important mechanism in the regulation of protein kinases in general, and there is much evidence suggesting that the activity of MLK proteins is also controlled in this manner. HPK has been shown to bind to, and phosphorylate MLK3 (Kiefer et al., 1996). MLKs have also been shown to be phosphorylated *in vivo*, and furthermore coexpression with a constitutively active cdc42 mutant (V12cdc42) alters the phosphorylation pattern and increases the *in vitro* catalytic activity of MLK3 (Bock et al., 2000), indicating that phosphorylation is an important mechanism of MLK regulation.

A recent study using phosphopeptide mapping and mass spectrometry analysis identified twelve potential MLK3 phosphorylation sites in total, most of which cluster at the C terminus (Vacratsis et al., 2002). It is important to emphasise, however, that these sites may not all be genuinely phosphorylated *in vivo*, although this result clearly emphasises the importance of phosphorylation in MLK regulation. The motif T T X X S (residues 277-281 of MLK3) is found in the putative activatory region of all MLKs, and mutagenesis studies support thr^{277} and ser^{281} as positive regulatory phosphorylation sites (Leung and Lassam, 2001; Durkin et al., 2004).

Interestingly, one of the potential MLK3 phosphorylation sites conforms to the consensus sequence for MAPK (including JNK) phosphorylation sites (P X S/T P), suggesting that JNK

might phosphorylate MLK3 in a potential feedback mechanism (Vacratsis et al., 2002). A similar feedback loop exists in the ERK pathway where the MAPK Raf is phosphorylated by ERK in response to insulin and growth factors (Lee et al., 1992). It has also been shown that JNK can phosphorylate the C-terminal region of MLK2, although no specific phosphorylation sites were determined (Sakuma et al., 1997). Transfection of dominant-negative JNK inhibits MLK2 phosphorylation in response to anisomycin-induced JNK activation (Sakuma et al., 1997). Further evidence for a positive feedback situation is provided by the observation that an MLK mutant lacking the C-terminal domain is sufficient to activate JNK, but the resulting apoptotic response is abrogated (Sakuma et al., 1997).

1.3.3.1 MLK activation by Rho family GTPases

The Ras-related Rho family of GTPases are small monomeric G proteins that function as a simple biochemical switch by cycling between two conformational states; a GTP-bound active conformation, and a GDP-bound inactive state. In the active state, these GTPases recognise and activate target proteins until their GTPase activity results in GTP hydrolysis and auto-inactivation. Their activity is activated by GEFs (guanine nucleotide exchange factors), and terminated by GAPs (GTPase-activating proteins). The Rho family in mammals represents 23 gene products including members of the rac and cdc42 subfamilies, and collectively these proteins have been implicated in a huge number of cellular functions, including maintenance of morphology (Tapon and Hall, 1997), motility (Aepfelbacher et al., 1994), adhesion (Nobes and Hall, 1995; Braga et al., 1997), cell division (Dutartre et al., 1996) and proliferation (Olson et al., 1995), smooth muscle contraction (Hirata et al., 1992), and vesicular transport (Murphy et al., 1996; Lamaze et al., 1996).

Many receptors have been implicated in the activation of Rho GTPases, including receptors for chemoattractants, cytokines, phagocytic particle uptake, growth factors, and recently TLRs. (Bokoch, 2005).

The CRIB (cdc42 and rac Interactive Binding region), characteristic of the MLK subfamily of proteins, was first identified by Burbelo et al (Burbelo et al., 1995) in the PAK-1 MAP3K. A functionally diverse repertoire of proteins possess CRIB domains and hence act as cdc42 and rac effector proteins. These include kinases such as the PAK and MLK families, but also proline-rich and SH3-containing adaptor proteins. The number and range of potential effectors for cdc42 and rac is perhaps unsurprising given the many functions with which the G-proteins have been associated. That cdc42 and rac activate MLK3 was first established by Teramoto et al (Teramoto et al., 1996) providing a pathway for JNK activation by these G-

proteins. Two cdc42-inducible phosphorylation sites in MLK3 have been identified subsequently although it is unclear how cdc42 induces MLK3 phosphorylation since it lacks kinase activity, and other kinases must be involved.

The p21-cdc42/rac-activated Kinase (PAK) family of MAP3K have emerged as the model CRIB-containing kinases and the focus of the majority of the research in this area, and as such, examination of the mechanism of PAK-cdc42/rac binding provides clues to the mode of activation of the MLK subfamily. The CRIB domains of MLK and PAK differ slightly (Burbelo et al., 1995), and therefore might differ in function. However mutation of the MLK3 CRIB disrupts the ability of this protein to bind cdc42, indicating that this is indeed a functional CRIB motif.

Bock et al (Bock et al., 2000) provide an interesting insight into the mechanism of MLK3 activation by cdc42. They observed that when MLK3 is in the resting, unphosphorylated state, the two proteins can be coprecipitated. However, following MLK3 phosphorylation, cdc42 is no longer detected. This suggests that upon activation by cdc42, MLK3 has a reduced affinity for the GTPase. In an exactly analogous fashion, the affinity of PAK-2 for cdc42 decreases following activation (Manser et al., 1995). Furthermore, the residues phosphorylated in MLK3 in response to cdc42 stimulation are exactly the same as those of PAK-2 (Bock et al., 2000), as judged by tryptic phosphopeptides.

Cdc42 is geranylgeranylated (Maltese and Sheridan, 1990) resulting in its localisation to cellular membranes and cytoskeletal elements (Erickson et al., 1996); it is therefore possible that cdc42 recruits MLK3 to the vicinity of an activating kinase resulting in phosphorylation and hence activation. This mechanism is reminiscent of the activation of another MAP3K, Raf, which in being activated by ras, clusters at the plasma membrane and becomes a target for other membrane kinases (Stokoe et al., 1994).

1.3.3.2 Dimerisation and autophosphorylation

In addition to phosphorylation by activatory kinases, there is evidence that MLK phosphorylation can also occur via dimerisation and autophosphorylation (Leung and Lassam, 2001). Indeed, the most striking feature of all MLK proteins is their ability to dimerise - suggesting that this is an important feature of their catalytic cycle. In addition to the leucine zipper domain characteristic of MLK proteins, all contain a second dimerisation mechanism: MLK1-3 possess both an SH3 domain and its substrate proline-rich region, the DLKs have a

second leucine zipper region and the ZAKs a sterile α motif, however, the significance of this is not as yet clear.

The importance of leucine zipper dimerisation to MLK activity was first illustrated by Leung and Lassam (Leung and Lassam, 1998) who showed using immuno-precipitation and non-reducing SDS PAGE that MLK3 forms homodimers that are stabilised by disulphide bonds, and that the leucine-zipper region is both necessary and sufficient for this homodimerisation. The precise mechanism by which leucine zipper-mediated dimers are reinforced by disulphide linkages, and the importance of these covalent bonds to the catalytic mechanism is not understood.

MLK3 proteins autophosphorylate following homodimerisation (Leung and Lassam, 2001), via a mechanism analogous to that of receptor tyrosine kinases (with which MLKs share sequence homology). Dimerisation is necessary for autophosphorylation because kinase-dead MLK3 can homodimerise but not autophosphorylate, whereas MLK3 minus the leucine zipper region lacks autophosphorylation activity. JNK is not activated by the MLK3 leucine-zipper deficient mutant suggesting that homodimerisation and autophosphorylation are critical steps in the catalytic mechanism of MLK3 (Leung and Lassam, 1998).

Some of these effects may have been because of the large protein structural alteration induced by domain deletion. To overcome this problem, using site directed mutagenesis, Vacratsis et al (Vacratsis and Gallo, 2000) introduced proline residues into the leucine zipper of MLK3 to disrupt the α-helical conformation with minimal protein structure disruption. They found that cdc42 was still able to activate this MLK3 mutant suggesting that dimerisation is not required for MLK3 activation by cdc42. Furthermore, Vacratsis et al report that the MLK3 mutant retains a high autophosphorylation capacity (Vacratsis and Gallo, 2000). This is an intriguing result because although it supports the idea that monomeric MLK3 binds to active cdc42, in this scenario dimerisation cannot subsequently occur, and yet autophosphorylation still results. Coexpression of cdc42 and MLK3 leads to a substantial increase in MLK dimerisation suggesting that cdc42 catalyses MLK3 dimerisation (Leung and Lassam, 1998). Taken together, these studies suggest that by binding to membrane-localised cdc42, the local concentration of MLK3 is increased sufficiently to allow autophosphorylation to occur in the absence of zipper-mediated dimerisation and disulphide reinforcement. Dimerisation is therefore not a prerequisite for autophosphorylation, but presumably increases the probability of fruitful MLK-MLK interaction.

The idea that cdc42 and rac increase the local concentration of MLK2 is also supported by the observation that when overexpressed, MLK2 is constitutively active (Nagata et al., 1998). Presumably in this situation the local concentration does not need amplification by membrane localisation for homodimerisation and autophosphorylation to occur. In support of this interpretation, this constitutive MLK activation is unaffected by cotransfection with constitutively active or dominant negative cdc42 or rac (Nagata et al., 1998).

1.3.3.3 SH3 domain

As well as the CRIB domain, MLK1-4 are also different from other MLKs in possessing an N-terminal SH3 domain, an independently folding domain of about 60 amino acids which functions to bind proline-rich polypeptides in a consensus sequence P X X X P preceded or followed by several basic amino acids.

The SH3 region of MLK3 has been shown to bind Haematopoietic Progenitor Kinase 1 (HPK-1) (Leung and Lassam, 2001), although the physiological significance of this is unclear (Kiefer et al., 1996). It was recently shown that the SH3 domain in MLK3 binds to a non-consensus SH3 binding site situated between the leucine-zipper and CRIB motifs. Point mutations introduced into this sequence or the SH3 domain increased the activity of the protein. Hence MLK3 is capable of negative regulation of its own activity (Zhang and Gallo, 2001). This was shown to be a specific autoregulation and not an inhibition by other SH3-containing proteins because an MLK3 with a mutated SH3 domain bound with much higher avidity to a GST-SH3 reporter. This suggests that in wild-type MLK3, intramolecular SH3 binding out-competes the reporter protein (Zhang and Gallo, 2001).

A similar mechanism of autoinhibition is observed in the Src family, where an intramolecular SH3-mediated association prevents ATP binding to the catalytic domain of the tyrosine kinase (Williams et al., 1998). Interestingly, in Src, the SH3 binds to the same non-consensus sequence as in MLK3. It is probable that this sequence induces a low affinity interaction because a more stable binding would prevent the release of autoinhibition. MLKs1, 2 and 4 also posses this binding sequence suggesting that all members of the MLK subfamily are regulated in this manner.

1.3.3.4 Auto-inhibition

The mechanism of release of autoinhibition has not been elucidated. One speculation was that higher affinity ligands for the SH3 domain, such as HPK-1, might induce release of the

intramolecular association (Zhang and Gallo, 2001). An alternative mechanism becomes apparent however, when one considers that in addition to the kinase domain, the leucine zipper also lies between the SH3 and its proline-rich binding region. Furthermore, the CRIB domain is adjacent to the proline-rich region and therefore this intramolecular bond may prevent MLK3 from dimerising or binding to cdc42 or rac. Activation of MLK3 may therefore involve the association of the CRIB domain with a GTP-bound G-protein resulting in displacement of the intramolecular SH3 bond, permitting homodimerisation via the leucine-zipper and autophosphorylation (figure 1.6). This model fits neatly with the observations outlined above regarding a model of cdc42 and rac increasing the local concentration of MLK3 prior to dimerisation. In this modified scenario cdc42 serves not only to increase local concentration but also to release the MLK autoinhibition. The concentration of activated MLK is therefore increased sufficiently such that MLK3 proteins can dimerise and autophosphorylate.

Adding further support to the auto-inhibition model, Mata *et al* showed that DLK also forms homodimers, obviously indicating an identical mechanism of autophosphorylation (Mata et al., 1996). Unlike MLK3 however, DLK does not contain a CRIB domain and hence cannot bind to the membrane-bound GTP forms of cdc42 and rac. Furthermore, DLK does not possess an SH3 binding region indicating that it is not capable of auto-inhibition, and therefore DLK doesn't require a G-protein interaction to activate it (Zhang and Gallo, 2001). DLK dimerisation does not require DLK phosphorylation or catalytic activity (Mata et al., 1996) and furthermore a protein corresponding to just the DLK leucine-zipper domain readily binds to itself or wild-type DLK (Nihalani et al., 2000). These observations suggest that the DLK-DLK interaction is of high affinity, and DLK is probably capable of spontaneous homodimerisation. The stimulus and regulation of DLK activity remains unclear, although it has been proposed that DLK is regulated by a sequestering protein.

1.3.3.5 Dimerisation and autophosphorylation revisited

With the auto-inhibition model in mind, one is forced to consider the question; if cdc42 is sufficient to induce autophosphorylation and activation of MLK monomers, then why is MLK dimerisation important?

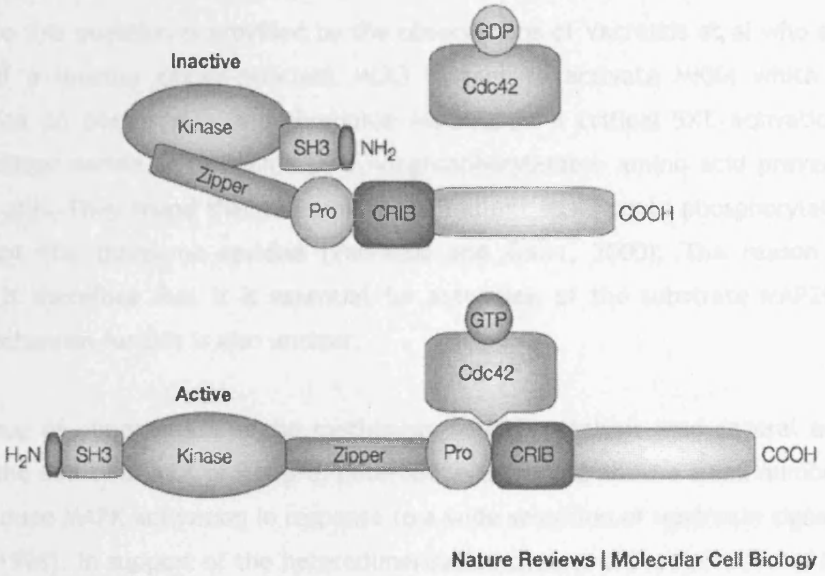

Figure 1.6. Autoinhibition of MLKs

It is proposed that autoinhibition of MLKs is achieved via binding of the amino-proximal SH3 domain to a proline (Pro)-rich sequence preventing activity of the kinase domain. Binding of activated (GTP-bound) Cdc42 or Rac to the CRIB domain is not compatible with simultaneous autoinhibitory interaction, thus the inhibition is released and the kinase domain of the MLK becomes catalytically competent. GTP hydrolysis is accompanied by reduced affinity of the GTPase for the MLK CRIB domain and hence dissociation and spontaneous reformation of MLK autoinhibition.

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Dimerisation clearly is important, however, since expression of a peptide corresponding to just the leucine zipper domain inhibits MLK3; obviously preventing dimerisation inhibits catalytic capability.

An insight into this question is provided by the observations of Vacratsis et al who examined the ability of a leucine zipper-deficient MLK3 mutant to activate MKK4 which requires phosphorylation on both serine and threonine residues in a critical SXT activation motif. Mutation of either serine or threonine to a nonphosphorylatable amino acid prevents MKK4 activation of JNK. They found that the monomeric mutant MLK3 could phosphorylate on the serine but not the threonine residue (Vacratsis and Gallo, 2000). The reason for MLK dimerisation is therefore that it is essential for activation of the substrate MAP2K, but in truth, the mechanism for this is also unclear.

The importance of dimerisation in the mechanism of MLK catalysis lead several authors to suggest that the ability of MLK proteins to heterodimerise would allow a small number of MLK proteins to induce MAPK activation in response to a wide selection of upstream signals (Leung and Lassam, 1998). In support of the heterodimerisation theory, coprecipitation of MLK2 and MLK3 was reported in this study. Further evidence is provided by Tanaka and Hanafusa (Tanaka and Hanafusa, 1998), who demonstrated interactions between MLK3 and DLK in 293T cells, and furthermore that kinase-dead DLK suppressed MLK3 activation of JNK, although they did not investigate the mechanism of this interaction. The authors interpret this as DLK lying downstream of MLK3 in the JNK activation pathway, but an alternative and more logical hypothesis in the light of the heterodimerisation model is that DLK and MLK3 bind via their leucine zippers. The catalytically inactive member would obviously be incapable of either autophosphorylation or substrate phosphorylation, resulting in termination of signal transduction.

However, this view is not universally accepted. In opposing the model of heterodimerisation, Nihalani et al (Nihalani et al., 2000) reported that DLK exclusively forms homodimers, and does not dimerise with the closely related LZK or more distantly related MLK3 proteins. They suggest that the inability of MLK proteins to heterodimerise is important to maintain signaling specificity and prevent cross-talk by limiting interactions between different MLK members in the same sub-cellular microenvironment.

1.3.3.6 JIP scaffolds

Research into MLK protein biology took a huge stride forward in 1998 with the discovery by Whitmarsh et al (Whitmarsh et al., 1998) of a mammalian MAPK scaffold protein, which they named JNK Interacting Protein-1 (JIP-1).

The JIP-1 is a homologue of scaffolds found in yeast such as Ste5p and Pbs2p, which coordinate components of the pheromone and osmoregulatory response pathways respectively. This initial investigation and a later study by the same group (Yasuda et al., 1999) identifying a homologue, JIP-2, determined that these scaffolds bind to JNK, MKK7 (but not the other JNK activating MAP2K, MKK4), MLK3 and DLK. It has since been shown that other MLK family members such as MLK2 and LZK also bind JIP-1 and -2, but MAP3Ks of other families such as MEKKs, Raf, MTK-1, PAKs, ASK-1 etc do not bind, suggesting that the role of the JIPs is to provide specificity of MLK family members for JNK activation (Nihalani et al., 2001; Ikeda et al., 2001a).

This model of a MAPK scaffold also potentially reconciles the contrasting theories regarding MLK protein heterodimerisation. Studies examining such heterodimerisation were based primarily on co-precipitating different MLK family members. However the discovery of JIPs and the observation that JIP-1 can multimerise leads to the possibility that different MLKs are bound on each JIP scaffold and in this case co-precipitation would result in detection of different MLK family members. With this in mind, and given the report by Nihalani et al that DLK does not dimerise with the closely related LZK protein, it is still controversial, but seems unlikely that heterodimerisation occurs between any of the MLKs.

Furthermore, a third interpretation of the data presented by Tanaka and Hanafusa is that the transfected kinase-dead DLK out-titrates active MLK3 and hence out-competes for binding to the JIP scaffold, thus inhibiting MLK3-induced JNK activation.

In addition, the identification of JIP scaffolds has focused attention on the mechanism of scaffold specificity. Using truncated constructs, Nihalani et al demonstrated that the N-terminal domain of DLK binds to JIP-1 and is necessary for JNK activation (Nihalani et al., 2001). This is in agreement with a study by Ikeda et al (Ikeda et al., 2001b) who found the analogous region on the closely related LZK protein to be important for binding to this scaffold.

Furthermore Ikeda et al report that a region of 14 amino acids entirely conserved between DLK and LZK in the C-terminal portion of the protein are essential for the activation of MKK4 but not MKK7 (Ikeda et al., 2001b). This is particularly interesting because like MKK7, MKK4 specifically activates JNK, however, unlike MKK7, MKK4 does not bind to all of the JIP scaffolds and MKK4-binding scaffolds are not as fully understood. A picture is therefore beginning to emerge of an MLK protein containing distinct functional regions, such as a JIP-binding domain in the N-terminus, a leucine zipper dimerisation domain, and specific discrete regions containing consensus-binding sequences for activation of different substrate kinases.

It is now known that four JIP scaffolds exist (Morrison and Davis, 2003). JIP-1 and -2 (also called IB-1 and -2) share sequence homology, as do JIP-3 (also called JSAP-1) and -4. The two functionally related pairs bear no structural similarities. The JIP proteins show different patterns of expression and slightly different MLK affinities, although all bind MKK7 and JNK. JIP-2 is unique in that it also binds MKK3 and p38 and has a higher affinity for p38 than JNK (Buchsbaum et al., 2002; Schoorlemmer and Goldfarb, 2001). JIP-3 and -4 bind MKK4 as well as MKK7 (Ito et al., 1999; Lee et al., 2002), and JIP-3 can also bind MEKK-1. MEKK-1 is a MAP3K which activates JNK (Yujiri et al., 1998) and has also been shown to activate ERK when overexpressed (Minden et al., 1994). MEKK-1 has also been shown to induce activation of the NF-κB transcription factor.

The scaffold proteins play a multi-regulatory role. Firstly they provide a mechanism for regulated activation of JNK by facilitating the sequential phosphorylation of the MAPK pathway, and secondly they insulate the pathway to prevent cross-talk with other MAPK systems transducing unrelated messages in the cytosol. JIP scaffolds have also been shown to bind phosphatases and therefore negatively regulate signal transduction (Willoughby et al., 2003). It is also thought that JIP scaffolds regulate the signaling pathways via subcellular localisation (see below).

1.3.3.7 JIPs and MAPK activation

There is much evidence to support the physiological importance of the JIP proteins. For example JIP has been shown to be critical for cytokine-induced apoptosis (Haefliger et al., 2003), and overexpression of the JNK binding domain of JIP-1 prevents apoptosis in sympathetic neurones (Harding et al., 2001).

Before the discovery of JIP scaffolds it was believed that MAPK were activated by a three-tier phosphorylation cascade to allow signal amplification. In other words, phosphorylation

induces kinase activation and each active enzyme could phosphorylate many substrate proteins. Mathematical models based on initial observations regarding levels of activated proteins appeared to support this amplification idea (Levchenko et al., 2000). However, while the model of MAPK enzymes bound to a scaffold protein in a "phospho-relay" assembly is attractive because signal specificity is highly regulated, the ability to amplify the signal is lost because amplification requires free diffusion of component kinases.

Recently, much work has investigated the nature of the interaction between MAPK pathway proteins and the JIP scaffolds, which may help to reconcile this conflict. Binding determinants have been identified mediating interactions at all levels of the pathway (Xu et al., 2001). JNK, MKK7 and MLKs bind to distinct regions of JIP-1 (Xu et al., 2001; Whitmarsh et al., 1998). Interestingly, studies using JIP-1 and DLK have suggested that the scaffold not only anchors the signaling proteins in the optimal orientation, but is also important in regulating the activity of the MAPK signal transduction.

Using a mutagenesis approach, binding sites within MKK7 and DLK were identified that were involved in mediating interaction with JIP-1. Surprisingly, however, it was also found that the leucine-zipper of DLK binds to a region in the N-terminal portion of MKK7. This suggests that dimerisation (and hence activation) of DLK, and binding to MKK7 may be mutually exclusive.

Furthermore it has been shown that DLK and JNK do not bind JIP simultaneously. Stimulation results in recruitment of JNK to the scaffold protein (Nihalani et al., 2001; Kelkar et al., 2000), and importantly, JNK recruitment coincides with decreased affinity of JIP for DLK. In the resting state, DLK is bound to JIP, and given the observation that in this state, MKK7 protein prevents DLK dimerisation and hence activation, DLK is therefore held in a monomeric, unphosphorylated and catalytically inactive state.

A model has been proposed whereby appropriate stimulation induces an increase in the affinity of JNK for JIP. This induces a decrease in the affinity of the scaffold for DLK, presumably by some subtle structural alteration of JIP. Upon release, DLK spontaneously homodimerises and becomes activated. The activated DLK dimer can then phosphorylate MKK7 and induce MAPK signal transduction (Nihalani et al., 2001).

The model solves two key problems. Firstly, it suggests that JIP is the sequestory protein responsible for preventing spontaneous DLK activation. Secondly, and more importantly, according to this scenario, activated DLK is not irreversibly bound to JIP and hence can

phosphorylate many MKK7 proteins. Thus, although the scaffold provides specificity, the observed signal amplification can still occur (Levchenko et al., 2000).

In addition to the data presented above regarding spontaneous DLK activation, other lines of evidence also support the model. Perhaps most important is that no-one has ever reported observing JIP bound to all three module components simultaneously. It is also reported that decreased JIP-DLK binding is observed in the presence of excess JNK or conditions that favour JNK activation. The affinity of activated DLK for MKK7 is sufficient for interaction to occur without stabilisation by a scaffold (Mata et al., 1996).

It is not clear, however, how JNK receives the initial signal to bind to JIP. Perhaps JNK activation occurs via an alternative MKK4-dependent system and the role of MKK7 and DLK is purely to amplify JNK activation. On the other hand, the system could work via the reverse mechanism to that described here. A stimulus might induce DLK release from JIP; this would have the dual effect of allowing spontaneous DLK activation, and would also increase the affinity of the JIP scaffold for JNK in readiness for MAPK signal transduction.

LZK shares a high degree of homology with DLK; it has also been shown to bind JIP-1 (Ikeda et al., 2001a), and hence is probably also regulated in this manner. However, while some features are surely consistent, this mechanism probably does not apply universally to all MLK family members. As outlined above, MLK3 has been shown to regulate its own activity negatively and has been reported to remain associated with JIP-1 following stress (Kim et al., 2002).

Other MLK-binding scaffold proteins have been reported including POSH (Plenty Of SH3s) which is thought to bind GTP-bound rac and mediate pro-apoptotic signaling via the JNK pathway (Tapon et al., 1998), because JNK-induced apoptosis is suppressed by POSH antisense oligonucleotides or siRNA (Xu et al., 2001). The POSH complex is negatively regulated by Akt-2, which induces complex dissociation (Figueroa et al., 2003). This is interesting because Akt-1 reduces the affinity of JNK for JIP-1 (Kim et al., 2002) suggesting that the Akt family might serve as negative regulators of JNK pathway signaling by disrupting scaffold complexes.

1.3.4 Subcellular localisation and MAPK-independent roles of MLKs

There have been several studies investigating the subcellular localisation of MLK proteins. The first of these, Nagata et al (Nagata et al., 1998) showed that MLK2 colocalises with dually phosphorylated JNK to discrete microtubule-associated structures. Before the discovery of JIP

scaffolds they also identified that MLK2 colocalises with two members of the kinesin superfamily of motor proteins. Later all four JIP proteins were shown to be cargoes for the motor protein Kinesin-1 (Whitmarsh et al., 2001; Bowman et al., 2000) and this protein probably controls subcellular localisation of MAPK activation. This corroborated the intracellular staining for JIP-1 and -2 proteins, which were found to accumulate close to the plasma membrane in cytoplasmic projections that extend from the cell surface (Yasuda et al., 1999). It is possible that the role of the kinesin motor is to localise the JIP-MAPK pathway cargo to the site of activation close to the cell membrane. Activated JNK phosphorylates its substrate c-jun in the nucleus, so following MAPK signal transduction, kinesin may carry deactivated JNK signalling components towards the plus end of microtubules from the nucleus to the cell periphery to re-prime the cells for signal transduction. JIP proteins may therefore mediate complex regulation of MAPK pathways via subcellular localisation and transport.

This model is strongly supported by Hirai et al (Hirai et al., 2002) who showed colocalisation of DLK to "dotted structures" along microtubules in a neuronal cell line. The DLK staining was dispersed along the length of the microtubules with many such structures seen at the tips of the microtubules towards the cell periphery.

MLK3 has also been reported to accumulate along microtubules, but primarily towards the minus ends at the centrosome (Swenson et al., 2003). This suggests that MLK3 might associate with a selection of different motor proteins resulting in control of subcellular localisation in a cell-type specific manner.

Alternatively, different MLK proteins may not just bind microtubules for localisation, but may also regulate some selective microtubule-associated cell processes. For example, Rasmussen et al (Rasmussen et al., 1998) have shown that MLK2 binds to the microtubule-associated GTPase dynamin, which controls synaptic vesicle transmission. Furthermore, a recent report suggests that MLK2 can interact with clathrin and regulate clathrin-coated vesicle transport (Akbarzadeh et al., 2002).

Based upon their colocalisation, Hirai et al suggest that DLK might be involved in controlling microtubule organisation (Hirai et al., 2002). Prior to DLK transfection, Cos-1 cells possessed radially organised microtubules that were completely disrupted upon DLK overexpression. Although DLK did not induce depolymerisation of tubulin, the microtubules became organised in a random fashion or accumulated at the cell periphery. Importantly, cells expressing a kinase-deficient DLK mutant did not show this altered microtubule pattern, and

microfilament organisation and formation of focal contacts were not affected by DLK overexpression. Hirai et al also studied neurones in the developing mouse cortex and reported that DLK expression was observed in a ring of cells in which migration is arrested. They conclude that expression of DLK is developmentally regulated and that the DLK-JNK MAPK pathway regulates neural cell migration in cortex development (Hirai et al., 2002).

Swenson et al (Swenson et al., 2003) also interpreted their findings in functional terms. They identified a region of human MLK3 in a search for proteins containing homology to an essential fungal cell cycle G2/M transition protein NIMA. They also found that MLK3 phosphorylation was observed at this stage of the cell cycle, although JNK activity was unaltered, suggesting that MLK3 has other functions independent of JNK. Providing further evidence for a role of MLK3 in cell cycle control, during the G2/M phase transition at which NIMA is known to be important, the subcellular localisation of MLK3 is transiently altered. Reminiscent of the study by Hirai et al, Swenson et al found that MLK3 localised to the centrosomal region and induced microtubule depolymerisation during G2/M transition (Swenson et al., 2003). The microtubule effects were not observed after expression of kinase-deficient MLK3 and silencing of MLK3 expression using siRNA resulted in increased sensitivity to the microtubule stabilising agent taxol, suggesting that endogenous MLK3 plays a physiological role in destabilising microtubules during M phase entry.

Several investigations support the role of MLK proteins in promoting cell proliferation. For example, overexpression of MLK3 induces growth of NIH 3T3 cells on soft agar (Hartkamp et al., 1999), and silencing of mlk3 by RNAi prevents serum-stimulated cell proliferation and the proliferation of tumour cells (Chadee and Kyriakis, 2004a). Furthermore, in support of the data presented by Swenson et al, it has recently been reported that inhibition of MLK proteins using the CEP11004 inhibitor blocked mitotic progression through a mechanism involving disruption of microtubule formation and spindle assembly (Cha et al., 2005). Interestingly, a region of the N-terminus of MLK2 has been shown to bind tubulin (Rasmussen et al., 1997) and may thus contribute to spindle formation. Although MLK proteins have therefore been investigated chiefly in dividing cells, MLK3 is also present in a wide spectrum of tissues including many cell types that are non-dividing. It is possible that in these cells MLK3 affects microtubule organisation during processes such as outgrowth and migration.

Hirai assumed that the cytoskeletal effects of DLK are mediated via JNK activation, but in the light of the Swenson report, it is possible that the MLK proteins have other functions than their MAP3K role (Hirai et al., 2002). In fact this was not the first demonstration of a JNK-

independent role for MLK3. A report by Hehner et al (Hehner et al., 2000) indicated that MLK3 is important in T cell stimulation-induced NF-κB activation. This is interesting because it suggests that one signal-transducing protein, MLK3, can induce activation of both transcription factors required for expression of a gene; in this case, c-jun (via JNK) and NF-κB transcription factors, and the IL-2 gene.

As described in section 1.2.6 (and figure 1.4), a cytoplasmic sequestory protein, IκB, controls NF-κB activity. IκB neutralisation is signalled by phosphorylation by an IκKinase Complex (IKC) containing IκB kinases, IKKα and IKKβ. These kinases are activated by IKKKs, all of which are also MAP3Ks and are attached to the IKC. Hehner et al show that MLK3 functions as an IKKK, and its activity is potentiated by the expression of other IKKKs, such as MEKK-1 (Hehner et al., 2000). However, MLK3-induced NF-κB activation was shown to be stimulus-specific, since CD3/CD28-induced NF-κB has been shown to be MLK3-dependent, whereas TNF-α and IL-1 activation of NF-κB is independent of MLK3 activity. For some time it has been known that rac and cdc42 are capable of activating NF-κB and therefore the identification of MLK3 as an IKKK obviously suggested a mechanism for this activation (Perona et al., 1997).

Further to the issue of spatial regulation of MLK proteins, it has recently been reported that JIP3 associates with TLR2, TLR4 and TLR9 in a mouse macrophage cell line (Matsuguchi et al., 2003). Obviously this makes good physiological sense because it optimises JNK activation in response to potential pathogens. None of the other JIP proteins have been reported to associate with TLRs, which is perhaps surprising, as one might have anticipated that a p38-binding scaffold would also be regulated in this manner. However, it may be that in this context, JIP is not just optimising JNK activation, but also acting to regulate NF-κB, a known effector of TLR stimulation. JIP3 has been shown to bind MEKK-1, a known IKKK that activates NF-κB. It is therefore probable that the true function of JIP3 in this situation is to localise MEKK-1 for optimal signal transduction from TLR to NF-κB.

An MLK3-deficient mouse has recently been reported (Brancho et al., 2005). Remarkably, despite the above evidence linking MLK proteins with important roles in the regulation of MAPKs and cell proliferation, the knockout mouse was viable and healthy. The only signaling phenotype observed was a defect in the ability of MEF cells to induce JNK activation in response to TNFα. Clearly an important regulatory role for MLK3 is not consistent with a phenotypically normal knockout mouse. This discrepancy may be due in part to functional reconstitution of MLK3 by other MLK proteins. However, many of the above reports are based upon MLK3 gene silencing using RNAi technology, and hence are more difficult to reconcile

with the results of the knockout study. Further investigations will therefore be required to fully elucidate the role of MLK3.

1.3.5 MLK inhibition

The importance of the MLK family in activating JNK and the role of this MAPK in neuronal cell death programs and many other degenerative diseases presents MLKs as a potential therapeutic target for drug development. A semi-synthetic MLK inhibitor, CEP1347, is currently in clinical trials for Parkinson's disease and has also shown potential therapeutic effects in models of Alzheimer's disease and hearing loss (Cheng et al., 2002).

CEP 1347 was developed following observations of the neurotrophic and survival promoting effects of K252a, a small molecule naturally produced by the *actinomycetes* group of gram-positive bacteria (Kase et al., 1986). K252a was initially observed to act as a relatively non-selective inhibitor of serine/ threonine kinases, but was later found to be a very potent inhibitor of the receptor tyrosine kinase TrkA. Specifically, by acting as a competitive agonist that binds to the ATP binding site, it blocks the autophosphorylation of TrkA with an IC_{50} of 3nM (Angeles et al., 1998) in response to neurotrophic ligands (Nye et al., 1992). Interestingly, however, despite inhibiting the action of neurotrophins, K252a was shown to possess its own "neurotrophic" activity. For example, neurones deprived of NGF were shown to survive for at least two weeks in culture in the presence of K252a (Borasio, 1990).

The drug was subsequently shown to exhibit these variable properties when present at different levels. At high concentrations, K252a inhibits neurotrophins, whereas at lower concentrations, K252a itself promotes survival. Compounds based on K252a were therefore synthesised to enhance the neurotrophic effects, while decreasing the inhibition of survival-promoting neurotrophins (Murakata et al., 2002). The 3, 9-bis-[ethylthio(methyl)]-substituted K252a was found to have the most potent neurotrophic effects and was subsequently named CEP1347 or KT-7515 (Murakata et al., 2002). CEP 1347 is not cytotoxic above 200nM, as is K252a, and does not possess the non-selective serine/ threonine kinase inhibition properties of K252a.

However, the true molecular target mediating the survival-promoting effects of CEP1347 was not known. In NGF-deprived neurones, CEP1347 was shown to prevent JNK activation (Xu et al., 2001), while having no inhibitory effects on other MAPKS (Maroney et al., 1999). The possibility of direct JNK inhibition by CEP1347 was excluded because MEKK-1-mediated JNK activation in CHO cells was insensitive to CEP1347 addition.

The ability of upstream regulators of JNK to induce c-jun phosphorylation in the presence or absence of CEP1347 was assayed by *in vitro* kinase assay (Xu et al., 2001). Direct activators of JNK were excluded because no inhibition of MAP2K-induced JNK activation was observed by addition of CEP1347.

Rac and cdc42 have been shown to signal NGF-deprivation-induced neuronal apoptosis (Bazenet et al., 1998) making them a candidate for CEP action. The G-proteins induced a 20-fold increase in JNK activity, and this activation was blocked by CEP1347, although no interaction between rac/cdc42 and CEP1347 could be demonstrated, indicating that CEP1347 acts downstream of these proteins.

MLK proteins were shown to be activated by rac and cdc42 (Mota et al., 2001; Xu et al., 2001) and induce JNK activation, suggesting that these could be the targets of CEP1347 action, and indeed the drug was shown to prevent MLK3-induced activation of JNK in CHO cells. CEP1347 was later shown to be highly specific for the MLK family, and inhibit these proteins with high potency (Murakata et al., 2002).

The specificity of CEP for MLK has been confirmed by genetic studies. For example cellular apoptosis induced by MLK transfection can be suppressed by CEP1347 treatment, (Xu et al., 2001) and dominant negative MLKs and CEP1347 suppress death induced by constitutively active rac1 and cdc42 (Xu et al., 2001).

With respect to the role outlined above of MLKs in microtubule organisation, one of the earliest papers regarding the initial biochemical characterisation of the K252a compound reported that not only did the drug mimic neurotrophins and promote survival, but it could also mimic growth factors and induce morphological change including neurite outgrowth. Such cellular alteration has not been mentioned again in any of the inhibitor studies. However in retrospect, an alternative explanation is that in the cells being studied, ciliary ganglion neurones, MLK proteins are involved in promoting microtubule instability. Inhibiting MLK therefore would induce microtubule polymerisation giving the appearance of outgrowth. Such an interpretation would be consistent with other observations of MLK-treated cells; for example Hirai et al observed loss of microtubule organisation in DLK-transfected neurones (Hirai et al., 2002), and Swenson et al suggest that MLK3 destabilises microtubules during prometaphase (Swenson et al., 2003).

In summary, MLK proteins, initially thought to be specific activators of the JNK pathway, may in fact be involved in various cellular processes. Comparatively little is known about these proteins, however, and almost all studies have been performed on neurones or neuronal cell lines. The MLK-specific inhibitors potentially provide a powerful tool for understanding MLK biology, in particular in non-neuronal cell death for which there are no reported studies, and in other physiological processes.

1.4 Aims of Thesis

1.4.1

Against the background outlined in the above introduction, the aim of the studies described in this thesis was to investigate the roles of MAPKs in DC physiology. Therefore the objective of the first part of the study was a comparative analysis of the roles of the JNK and p38 MAPK pathways in the response of DC to oxidative stress and LPS.

1.4.2

Based upon the observations made in section 1.4.1, the aim of the subsequent experiments was:

a. To investigate the expression and function of MLK proteins in the response of DC to selected TLR ligands.

b. To design and optimise a novel assay which permits investigation of the relationship between the morphology of DC and the ability of DC to internalise antigen.

Chapter 2

Materials and Methods

2.1 Materials and Reagents

2.1.1 Materials and equipment

All cell manipulation work was performed in a M.D.H. Intermed class 2 safety cabinet, using sterile techniques. Reagents were of the highest grade available. Pipette tips were from Elkay (Hampshire, UK). All culture plates, flasks, and centrifuge tubes were from Nunc (Rochester, NY, USA) unless otherwise stated.

2.1.2 Solvents

Where possible, stimuli and inhibitors were obtained in pure solid or lyophilised form to minimise decomposition and were then dissolved in the relevant solvent (table 2.1).

Table 2.1 Solvents

Ref	Name	Structure	Manufacturer	Cat. #
DiH_2O	Water for Molecular Biology	H_2O	Sigma Chemical Co. Poole, UK	W4502
DMSO	Dimethyl Sulphoxide	$CH_3-\overset{\overset{O}{\|}}{S}-CH_3$	Sigma	D8779
EtOH	Ethanol	CH_3CH_2OH	Sigma	E7148
HBSS	Hanks' Balanced Salt Solution	N/A	Invitrogen, Carlsbad, CA, USA	14175-053

2.1.3 Stimuli

All stimuli were added in a 1:100 ratio to keep cell and cytokine concentrations constant. Where applicable, stimuli were dissolved in the appropriate primary solvents (table 2.2) and then diluted to working concentrations in CM.

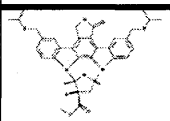
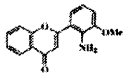
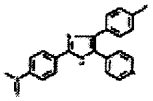
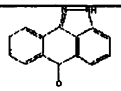
Table 2.2 Stimuli

Ref	Name	Origin	Solvent	Receptor	Manufacturer	Cat. #
FN	Fibronectin	Bovine Plasma	DiH_2O	N/A	Sigma	F1141
H_2O_2	Hydrogen Peroxide	N/A	N/A	Redox Sensitive Molecules	Sigma	H1009
LPS	Lipopolysaccharides	Salmonella minnesota (ATCC 9700)	CM	TLR4	Sigma	L6261
Poly (I:C)	Polyriboinosinic polyribocytidilic acid	N/A	CM	TLR3	Sigma	P1038
U.V.	Ultra Violet Light	N/A	N/A	N/A	N/A	N/A
Zym	Zymosan A	Saccharomyces cerevisiae	HBSS	TLR2/ TLR6 Dectin-1	Sigma	Z4250

2.1.4 Inhibitors

Inhibitors were dissolved in the appropriate primary solvents (table 2.3) and then diluted to working concentrations in CM.

Table 2.3 Inhibitors

Ref	Name	RMM	Solvent	Cellular Target	Structure	Manufacturer	Cat. #
CEP	CEP11004 (KT8138)	643.83	DMSO	MLK1, 2, 3, DLK		Cephalon, West Chester, PA, USA	N/A
Na_3VO_4	Sodium Orthovanadate	183.91	H_2O	Tyrosine Phosphatases	Na_3VO_4	Sigma	S6508
PD	PD98059	267.28	DMSO	MEK		Sigma	P215
SB	SB203580	377.43	DMSO	p38α, β		Sigma	S8307
SP	SP600125	220.23	DMSO/ EtOH	JNK1, 2, 3		Celgene, San Diego, CA, USA	N/A

2.2 Cell Culture

2.2.1 Media

The complete medium (CM) used comprised Roswell Park Memorial Institute medium (RPMI)-1640 (Gibco BRL, Paisley, UK) supplemented with penicillin (100U/ml), streptomycin (100μg/ml), L-glutamine (2mM) (all from Cancer Research UK Clare Hall Laboratories, London, UK) and foetal calf serum (FCS) (10% v/v) (Gibco BRL, Paisley, UK) (heat inactivated 56°C, 30 minutes) unless otherwise indicated.

2.2.2 Culture condition

Unless otherwise stated, all incubations at 37°C were carried out in an incubator at 5% CO_2. Incubations at 4°C were carried out on ice.

2.2.3 Cell counting

Cell numbers were measured using a Neubauer haemocytometer. 10μl of cell sample was mixed with an equal volume of 0.04% trypan blue stain (Gibco BRL, Paisley, UK), and viable cells counted by light microscopy.

2.2.4 Isolation of peripheral blood mononuclear cells

PBMC were isolated from healthy volunteers. Peripheral blood was obtained by venesection into a syringe containing approximately 50μl heparin (5000IU/ml, CP Pharmaceuticals, Rexam, UK). The blood was diluted into an equal volume of HBSS, and then layered over Lymphoprep 1077 (Nycomed Pharma, Oslo, Norway) and centrifuged for 30 minutes, 600g, at room temperature. The interface between aqueous and Lymphoprep layers containing the PBMC fraction was then aspirated and cells were washed three times with HBSS.

2.2.5 Derivation of monocyte-derived dendritic cells

The PBMC fraction was resuspended in CM and allowed to adhere to tissue culture plates (Falcon, Becton-Dickinson, Mountain View, CA, USA) for 2 hours. Non-adherent cells were then removed by washing with HBSS, leaving an adherent cell population of predominantly monocytes. These cells were then cultured in CM supplemented with 100ng/ml human recombinant GM-CSF and 50ng/ml IL-4 (both gifts from Schering-Plough Research Institute,

Kenilworth, New Jersey, USA) to induce differentiation into monocyte-derived dendritic cells (MDDC).

Loosely adherent cells were collected on day 4 of culture and layered over Lymphoprep 1077 (Nycomed Pharma, Oslo, Norway) as described above to remove dead cells and debris. Cells at the interface were washed in HBSS, and then the proportion of contaminating, non-MDDC cells was estimated by cell size by light microscopy. The cells were then incubated in CM at 4°C for 30 minutes in the presence of mouse antibodies raised against human CD2, CD3, and CD19 to bind to the major contaminating cell populations: NK cells, T cells, and B cells respectively (table 2.4). Excess antibody was removed by washing in cold HBSS and the cells resuspended in cold CM. Immunomagnetic beads coated in sheep anti-mouse IgG (Dynal, Merseyside, UK) were added (approximately 1 bead: 1 contaminating cell) and the mixture rotated for 45 minutes at 4°C. Bead-conjugated cells were removed by magnetic separation and the remaining free cells, which contained the purified MDDC were cultured for a further 3 days at 5 x 10^5 cells/ml in CM containing GM-CSF and IL-4. On day seven of culture, MDDC were removed and counted.

Table 2.4 Antibodies for Negative Selection of Monocyte-Derived Dendritic Cells

Target	Origin	Clone	Isotype	Manufacturer	Cat. #
Human CD2	Mouse mAb	RPA-2.10	IgG1	eBioscience, San Diego, CA, USA	14-0029
Human CD3	Mouse mAb	UCHT1	IgG1	Gift, Prof P. Beverley, The Edward Jenner Institute, Compton, UK	N/A
Human CD19	Mouse mAb	BU12	IgG1	Gift, D. Hardie, Birmingham Medical School, Birmingham, UK	N/A

2.2.6 293T Cells

293T cells were a gift from E. Willoughby and used to manufacture control lysates positive for activated p38 and JNK MAPK.

2.2.7 Storage of cell lines

For storage in liquid nitrogen, approximately 5 x 10^5 cells/ml were resuspended in FCS containing 10% (v/v) DMSO. For recovery, cells were rapidly returned to room temperature and transferred into a 25cm^2 flask containing the appropriate selection medium. Cell lines were regularly tested and shown to be negative for mycoplasma antigens.

2.3 Cell Visualisation and Analysis

2.3.1 Light microscopy

Cells were visualised on a Wilovert microscope.

2.3.2 Confocal microscopy

2.3.2.1 Cell preparation

For analysis by confocal microscopy, MDDC were washed twice in CM and plated on FN-coated (Sigma) glass coverslips at 3 x 10^4 DC per group in 0.5ml CM containing GM-CSF and IL-4. Cells were incubated for two hours to allow adherence, prior to treatment.

2.3.2.2 Microscopy

Cells were visualised on a Zeiss confocal microscope using BioRad Lasersharp software, and Adobe Photoshop Elements.

2.3.3 Flow cytometry

Cells were examined using a FACScan (Beckton-Dickinson) and for each sample at least 10,000 events were acquired. Data were analysed using WinMDI software (Joseph Trotter, Scripps Research Institute), and the median fluorescence intensity (MFI) was used for quantitative analysis of bound antibody levels.

2.4 Analysis of Cell Viability

Several different and complementary assays of cell viability were used.

2.4.1 Trypan blue analysis

Cells were counted using trypan blue stain as outlined above. This technique allowed a rapid assessment of cell viability, since trypan blue is excluded from live cells.

2.4.2 MTT reduction

Cell viability was assessed by monitoring reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 100µl of 10^5 purified DC per group were incubated for 24 hours in 96-well plates (Nunc, Roskilde, Denmark). 20µl of MTT (final concentration 1mg/ml; Sigma) in PBS was added to triplicate samples and incubated at 37°C, 5% CO_2 for 4 hours. The reduction of MTT was stopped by the addition of 100µl of 10% w/v sodium dodecyl sulphate (SDS) (Sigma). Absorbance was measured after a further 18 hours incubation in the dark. Optical absorbance was measured at a wavelength of 570nm with wavelength correction at 630nm.

2.4.3 Analysis of cell size and granularity

Cells were analysed by flow cytometry as described above and the laser deflection data plotted according to Forward Scatter (FSC), and Side Scatter (SSC), which give an indication of cell size and granularity respectively. FSC/SSC parameters of healthy cells were defined and the percentage of cells from treated samples possessing these parameters was calculated.

2.4.4 PI staining

Apoptosis was assessed by measuring cellular DNA content following disruption of cell membrane integrity. For permeabilisation, $5x10^5$ DC per group were resuspended in 1ml of 70% ethanol at -20°C and stored on ice for 30 minutes. The cells were then washed twice and treated with DNase-free RNase A (Sigma) (final concentration 500µg/ml) at 37°C for 10 minutes. To stain DNA, Propidium Iodide (PI) (Sigma) was added (final concentration 50µg/ml), and the cells were analysed immediately by flow cytometry.

2.4.5 Annexin V/ PI staining

For Annexin V/ PI experiments, $2x10^5$ DC per group were stained using the TACS™ Annexin V-FITC apoptosis detection kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions prior to analysis by flow cytometry. Briefly, cells were washed in HBSS and resuspended in 100µl of full staining buffer for 30 minutes. In parallel, control groups were prepared by resuspending in 100µl of Annexin V control buffer, PI control buffer, or negative control buffer. Cells were washed, resuspended in 1x binding buffer, and then immediately analysed by flow cytometry.

Table 2.5 Buffers for Annexin V/ PI Staining

Buffer	Constituents	Manufacturer
10x Binding Buffer	100mM HEPES pH 7.4 (pH with NaOH)	Sigma
	1.5M NaCl	Sigma
	50mM KCl	Sigma
	10mM $MgCl_2$	Sigma
	18mM $CaCl_2$	Sigma
Full Staining Buffer	1x Binding Buffer	
	PI 5µg/ml	R&D Systems
	Annexin V 0.5µg/ml	R&D Systems
	DiH_2O	Sigma
Annexin V Control Buffer	1x Binding Buffer	
	Annexin V 0.5µg/ml	R&D Systems
	DiH_2O	Sigma
PI Control Buffer	10x Binding Buffer	
	PI 5µg/ml	R&D Systems
	DiH_2O	Sigma
Negative Control Buffer	1x Binding Buffer	R&D Systems
	DiH_2O	Sigma

2.5 Detection of Secreted Proteins

2.5.1 Cytometric bead array

DC were incubated at 5 x 10^5 cells per group before collection of supernatants and cytokine concentration determination by cytometric bead array (Beckton-Dickinson) according to manufacturer's instructions. Briefly, 10µl of the provided mixed capture bead suspension and 50µl of PE detection reagent were added to 50µl of sample and incubated for 3 hours. In parallel, dilutions of cytokine standards of known concentration were also prepared. Samples were then washed with the provided wash buffer and then examined using a FACSArray Flow cytometer (Beckton-Dickinson) and analysed with BD CBA software (Beckton-Dickinson).

2.5.2 Data and statistical analysis

Where appropriate, to facilitate comparison, concentrations of secreted cytokines were adjusted such that the level of the control-treated group was indicated as 100%.

Statistical significance of changes in the level of secreted cytokine was determined using a paired student's T-test (on the raw data and not the adjusted figures).

2.6 Detection of Surface Proteins

2.6.1 Extracellular immunofluorescence staining

For phenotypic analysis, 5 x 10^4 - 2 x 10^5 cells per group were harvested (within individual experiments the number of cells in each group was the same to ensure a constant cell: antibody ratio). Cells were resuspended in 100µl of the appropriate blocking buffer for 15-30 minutes at 4°C.

Table 2.6 Buffers for Immunofluorescence Staining

Lysis Buffer	Constituents	Manufacturer
Rabbit Blocking Buffer (Mouse Primary Antibodies)	HBSS NaN_3 0.1% Rabbit Serum 10%	Invitrogen Sigma Gibco BRL
FCS Blocking Buffer (Rabbit Primary Antibodies)	HBSS NaN_3 0.1% Foetal Calf Serum 10%	Invitrogen Sigma Gibco BRL
Washing Buffer	HBSS NaN_3 0.1%	Invitrogen Sigma
Fixation Buffer	HBSS Formaldehyde 3.7% (v/v)	Invitrogen BDH, Poole, UK

50µl of the appropriate primary antibody (table 2.7) were mixed with the cell suspension in a 96-well round bottomed plate (Nunc), and then incubated for a further 30 minutes at 4°C. For experiments where the primary antibodies were not directly conjugated to fluorochromes, cells were washed twice with the appropriate blocking buffer to remove excess primary antibody, and then incubated for 30 minutes at 4°C with the relevant secondary antibody diluted in blocking buffer.

Finally, cells were washed twice in washing buffer and then fixed by resuspension in 50µl washing buffer followed by 100µl fixation buffer. Samples were stored at 4°C in the dark, and then analysed by flow cytometry as outlined above.

2.6.2 Data and statistical analysis

Changes in median fluorescence intensity were adjusted to 100% for comparison purposes, and to investigate statistical significance, raw data was analysed by paired student's T test.

Table 2.7 Primary Antibodies for Immunofluorescence Staining

Target	Conjugate	Origin	Clone	Isotype	Manufacturer	Cat. #
Human CD1a	Unconjugated	Mouse mAb	NA1/34	IgG2a	Gift, Prof. A. McMichael, John Radcliffe Hospital, Oxford, UK	N/A
Human CD3	Unconjugated	Mouse mAb	UCHT1	IgG1	Gift, Prof P. Beverley	N/A
Human CD11c	Unconjugated	Mouse mAb	ICRF 319	IgG1	Gift, Prof N. Hogg, Cancer Research UK, London, UK	N/A
Human CD13	FITC	Mouse mAb	WM15	IgG1	DPC Biermann, Bad Nauheim, Germany	SM1070F
Human CD14	Unconjugated	Mouse mAb	HB246	IgG2b	Gift, Prof. P. Beverley	N/A
Human CD19	Unconjugated	Mouse mAb	BU12	IgG1	Gift, D. Hardie	N/A
Human CD25	PE	Mouse mAb	B1.49.9	IgG2a	IO Test, Beckman Coulter	0497
Human CD36	Unconjugated	Mouse mAb	SM0	IgM	Cymbus Biotechnology, Southampton, UK	CBL 168
Human CD54	Unconjugated	Mouse mAb	HA58	IgG1	eBioscience	14-0549
Human CD80	PE	Mouse mAb	MAB104	IgG1	IO Test, Beckman Coulter	IM1976
Human CD83	Unconjugated	Mouse mAb	HB15e	IgG1	Dako Cytomation	LS109
Human CD86	Unconjugated	Mouse mAb	BU63	IgG1	Gift, D. Hardie	N/A
Human MHC I	Unconjugated	Mouse mAb	W6/32	IgG2a	Serotec, Oxford, UK	MCA81
Human HLA-DR	Unconjugated	Mouse mAb	L243	IgG2a	Gift, Prof. P. Beverley	N/A

Table 2.8 Secondary Antibodies for Immunofluorescence Staining

Target	Conjugate	Origin	Clone	Isotype	Manufacturer	Cat. #
Mouse IgG	FITC	Rabbit	Polyclonal		Dako Cytomation,	F0261
Mouse IgG	PE	Goat	Polyclonal		Dako Cytomation,	R0480

Table 2.9 Isotype Control Antibodies for Immunofluorescence Staining

Target	Conjugate	Origin	Clone	Isotype	Manufacturer	Cat. #
Isotype Control	PE	Mouse mAb	679.1Mc7	IgG1	IO Test, Beckman Coulter, Fullerton, CA, USA	IM0670
Isotype Control	PE	Mouse mAb	DAK-GO5	IgG2a	Dako Cytomation, Glostrup, Denmark	X0950
Isotype Control	FITC	Mouse mAb	DAK-GO1	IgG1	Dako Cytomation	X0927
Human CD2	Unconjugated	Mouse mAb	MAS 593	IgG2b	Harlan Sera-lab, Crawley Down, UK	N/A
Human CD19	Unconjugated	Mouse mAb	BU12	IgG1	Gift, D. Hardie	N/A
Isotype Control	Unconjugated	Mouse mAb	HB38	IgM	Prof. B. Chain	N/A
Isotype Control	Unconjugated	Mouse mAb	DAK-GO5	IgG2a	Dako Cytomation	X0943

2.7 Detection of Intracellular Proteins

2.7.1 Sample preparation

MDDC were incubated in CM containing 0.5% FCS v/v for at least 24 hours prior to treatment. Total cell extracts were prepared by resuspending the cells in 50μl of reducing sample buffer (table 2.10) at approximately 5 x 10^6 cells/ ml. Where necessary, samples were stored at -20°C, and were sonicated and boiled for 5 minutes prior to gel loading. Samples were then analysed by polyacrylamide gel electrophoresis.

Table 2.10 Lysis Buffers

Lysis Buffer	Constituents	Manufacturer
Reducing Sample Buffer	60mM Tris pH 6.8	Sigma
	10% Glycerol	Calbiochem, CA, USA
	2% SDS	BDH
	Bromophenol Blue	Sigma
	2% 2-Mercaptoethanol	Gibco

2.7.2 Control lysates

293T cells were irradiated (positive control lysate) or not (negative control) with UV light for 60 seconds and then incubated at 37°C for a further 20 minutes. Cells were then washed, lysed in reducing sample buffer, sonicated, and stored in aliquots at -20°C. Control lysates were boiled prior to use.

Table 2.11 Western Blotting Buffers

Buffer	Constituents	Manufacturer
Running Buffer	192mM Glycine	Sigma
	25mM Tris-base	Sigma
	0.1% SDS	Sigma
Transfer Buffer	192mM Glycine	Sigma
	25mM Tris-base	Sigma
	20% methanol	Sigma

2.7.3 Gel running and analysis

Within individual experiments, a consistent volume of between 10μl and 40μl of lysate was resolved by running on a 12.5% polyacrylamide gel and transferred to ECL nitrocellulose

membrane (Amersham Pharmacia Biotech, Bucks, UK) for analysis by immunoblotting. Buffers for electrophoresis and migration of proteins to nitrocellulose membranes are indicated in table 2.11.

2.7.4 Immunoblotting

Following transfer of electrophoresed lysates, nitrocellulose membranes were washed in TBS (table 2.12) and then incubated in blocking buffer for 1 hour at room temperature or overnight at 4°C. Membranes were then washed in TTBS prior to incubation with the primary antibody under the conditions indicated in table 2.13. The process of washing and incubation with antibody buffer was repeated for the secondary antibody (table 2.13). ECL detection reagent and ECL hyperfilm (both Amersham Pharmacia Biotech) was used to visualise the membranes.

Where necessary, membranes were stripped of bound antibodies by incubating at 60°C for 1 hour in stripping buffer (table 2.12). Membranes were then washed at least 5 times in TTBS before immunoblotting was performed as described above.

Table 2.12 Buffers for Immunoblotting

Buffer	Constituents	Manufacturer
TBS	NaCl KCl Tris pH 7.5	Sigma Sigma Sigma
TTBS	TBS 0.1% Polyoxyethylene sorbitan monolaurate (Tween-20)	Sigma
Blocking Buffer	TTBS 5% Skimmed low fat milk powder	Tesco, Cheshunt, Hertfordshire, UK
Milk Antibody Buffer	TTBS 1% Skimmed low fat milk powder	Tesco
BSA Antibody Buffer	TTBS 5% Bovine Serum Albumen	Sigma
Stripping Buffer	2% SDS 60mM Tris pH 6.7 2% 2-Mercaptoethanol	BDH Sigma Sigma

Table 2.13 Antibodies for Immunoblotting

Target	Buffer	Conjugate	Origin	Clone	Isotype	Manufacturer	Cat. #
Primary Antibodies							
Total p38	Milk 20°C	Unconjugated	Rabbit	Polyclonal		Cell Signaling Technology, Beverly, MA, USA	9212
Phospho p38	Milk 20°C	Unconjugated	Rabbit	Polyclonal		Cell Signaling Technology	9211
Total JNK	Milk 20°C	Unconjugated	Mouse mAb	N/A	IgG1	Santa Cruz Biotechnology	sc-7354
Phospho JNK	Milk 20°C	Unconjugated	Rabbit	Polyclonal		Promega, Madison, WI, USA	V9731
Phospho ERK	BSA 4°C	Unconjugated	Mouse mAb	N/A	IgG1	Cell Signaling Technology	9106
Total c-jun	Milk 20°C	Unconjugated	Rabbit	Polyclonal		Santa Cruz Biotechnology	sc-1694
Phospho c-jun	Milk 20°C	Unconjugated	Mouse mAb	N/A	IgG1	Santa Cruz Biotechnology	sc-822
Phospho tyrosine	Milk 20°C	Unconjugated	Mouse mAb	4G10	IgG2b	Upstate Biotech, NY, USA	05-321
Total IκBα	Milk 20°C	Unconjugated	Rabbit	Polyclonal		Santa Cruz Biotechnology	sc-371
Phospho IκBα	Milk 20°C	HRP	Mouse mAb	N/A	IgG2b	Santa Cruz Biotechnology	sc-8404
Total MLK3	BSA 4°C	Unconjugated	Rabbit	Polyclonal		Cell Signaling Technology	2187
Phospho MLK3	BSA 4°C	Unconjugated	Rabbit	Polyclonal		Cell Signaling Technology	2811
Secondary Antibodies							
Mouse IgG	Milk 20°C	HRP	Rabbit	Polyclonal		Dako Cytomation	P0260
Rabbit IgG	Milk 20°C	HRP	Swine	Polyclonal		Dako Cytomation	P0399

2.7.5 Densitometry and analysis

Densitometric analysis of autoradiographs was performed using GeneSnap software (Syngene). Densities of the Phospho- and Total-protein bands were calculated and the ratios of Phospho to Total densities were then calculated to give an indication of the proportion of kinase phosphorylated in each condition.

2.8 Detection of Messenger RNA

2.8.1 Polymerase chain reaction

2.8.1.1 Genomic DNA preparation

5 x 10^6 immature DC were resuspended in 500µl lysis buffer (table 2.14) at 55°C with shaking overnight. Genomic DNA was then precipitated using propan-2-ol (BDH, Poole, UK), and resuspended in dH_2O (Sigma) prior to concentration determination by spectrophotometry and storage at -20°C.

Table 2.14 Lysis Buffers

Lysis Buffer	Constituents	Manufacturer
Lysis Buffer (for DNA Prep)	100mM Tris pH 8.5	Sigma
	5mM EDTA	Sigma
	0.2% SDS	BDH
	200mM NaCl	Sigma
	1% Proteinase K	Sigma

2.8.1.2 Total RNA preparation

5 x 10^6 immature DC were lysed using TRIzol (Invitrogen) and RNA was collected by addition of 20% chloroform (BDH) and centrifugation (12,000g/ 10 min/ 4°C). The top (RNA containing) layer was collected and the chloroform addition and centrifugation procedure repeated. RNA was then cleaned by sequential addition, centrifugation (12,000g/ 10 min/ 4°C) and supernatant removal of propan-2-ol and ethanol (twice; BDH). Contaminating DNA removed by resuspension in DNase buffer (Promega, Promega, Madison, WI, USA) at 37°C for 1 hour. RNA was extracted by addition of PCI (Phenol, chloroform, isoamylalcohol (24:24:1 ratio; BDH)), centrifugation (13,000rpm/ 10 min) and top layer collected. This process was repeated twice and finally an equal volume of chloroform was added. RNA was precipitated by addition of 8M ammonium acetate (Sigma) followed by ethanol addition and RNA precipitation (repeated three times). Finally RNA was resuspended in dH_2O prior to concentration determination by spectrophotometry and storage at -20°C.

2.8.1.3 Reverse transcription and PCR

Oligo-dT (Promega) was added to 5μg RNA and incubated at 70°C for 10 mins prior to addition of RT mixture (AMV 5x buffer, dNTPs, 5% AMV RT (all Promega), 10% DTT (Sigma)), incubation at 42°C for 50 mins and then 70°C for 15 mins.

2.8.2 PCR primers

To maximise stringency and sensitivity of detection of MLK protein synthesis, a nested strategy was used whereby one or two primers were designed to be used in a second PCR reaction using the first round product as template. The sequences of all the all primers used are given in table 2.15.

Table 2.15 Primers for detection of MLK Proteins

Name	Sequence (5'-3')
FMLK1	CTTGCTGGCAGCCAGTTG
RMLK1	ATCTCGTTTGAAGCGCTC
FnestMLK1	TGGTGCCCATCGACATTG
RnestMLK1	GACCCTCCCGTCTTTTCTTC
FMLK2	TGGAGCTGGAGAGCTTCAAGAAG
RMLK2	GGAAGTCCAGAAGGTCACGA
FnestMLK2	CAGTCGCTCACGCCCACCCACG
FMLK3	AGCCCATTGAGAGTGACGAC
RMLK3	CGTCGAAGAGACCCTGGAT
FnestMLK3	AGTGGCACAAAACCACACAA
RnestMLK3	CTGCATGGAATGGAAGGAGT
FMLK4	TGGAGTGCTGCTGTGGGA
RMLK4	CTTTTCCTTTGTTCTCAACTC
RnestMLK4	GTTTCCAGTCATCTTGCATGG
FDLK	AGTGGCACAAAACCACACAA
RDLK	TCAACGCTGTTGGAGTTGTC
FnestDLK	TAGTGAACCTTCCCCCAGTG
BactinF	GACGAGGCCCAGAGCAAGAGACG
BactinR	GATCCACATCTGTGGAAGGTGGAC

2.8.3 PCR conditions

Table 2.16 PCR Conditions

Reagent	Concentration	Source	Volume (Total 50µl)
PCR Buffer	10x	Promega	10µl
$MgCl_2$	25mM	Promega	2µl
dNTPs	2.5mM (each dNTP)	Promega U1240	1µl
DMSO	100%	Sigma	2µl
Taq Polymerase	5u/µl	Promega M1665	0.5µl
DiH_2O	-	Sigma W4502	33.5µl
Template	gDNA 0.3µg/µl cDNA 0.8µg/µl		2µl
Primers	10µM	Sigma Genosys	2µl

2.8.4 PCR program

Table 2.17 PCR Program

Temperature	Duration	Repeat
94 (°C)	2 minutes	x1
94 (°C)	1 minute	x35
58 (°C)	1 minute	
72 (°C)	2 minutes	
72 (°C)	2 minutes	x1

2.9 Quantification of Antigen Internalisation

2.9.1 Uptake of FITC-conjugated dextran

5 x 10^4 MDDC were allowed to adhere to FN-coated glass coverslips as described above and treated overnight as indicated. FITC-conjugated dextran was prepared in CM containing GM-CSF and IL-4 and added to the cells to a final concentration of 30µg/ml. Cells were then incubated at 37°C or 4°C as appropriate for 90 minutes. Cells were removed by five-minute treatment with trypsin and were then washed three times in washing buffer (table 2.6). Cells were fixed in 100µl of fixation buffer (table 2.6) and incubated at 4°C in the dark prior to analysis by flow cytometry.

2.9.2 Preparation of microbeads

The experimental procedure is outlined in figure 5.4. Beads coated with polyclonal sheep anti-rat IgG antibodies (Dynal) were used at 2 x 10^5 per group (approximately 4 beads: 1 cells).

Beads were washed once in HBSS and resuspended in 200µl of CM containing 5µl of PE-conjugated rat anti-human DC-SIGN antibody (final concentration 3µg/ml), or isotype control antibody (specificity unknown, 0.2mg/ml). Following agitation for one hour at 4°C, beads were washed to remove excess antibody and resuspended in 100µl of CM (containing GM-CSF and IL-4) and then added to the cells.

Beads coated with PE-conjugated antibodies raised against CD80, CD83, MHC class I, and CD1a were treated in exactly the same way, except that rabbit anti-mouse IgG coated beads (Dynal), and FITC-conjugated rabbit anti-mouse secondary antibodies (Dako) were used.

2.9.3 Analysis of microbead internalisation

To analyse the ability of MDDC to internalise microbeads, following treatment, the medium was carefully removed and replaced with 100µl of microbeads in CM prepared as described above.

After incubation under the indicated conditions and time period, the medium was removed carefully and replaced with 500µl of fixation buffer (table 2.6).

2.9.4 Determination of internal versus external microbeads

To distinguish between internalised (protected) and externally bound (accessible) microbeads, a second round of staining was performed (see figure 5.4). For secondary staining, formaldehyde was removed and cells washed twice in washing buffer (table 2.6) prior to incubation for one hour at 4°C in 500µl of Rabbit Blocking Buffer (table 2.6). To this was added 100µl of FITC-conjugated rabbit anti-rat antibody (4mg/ml) diluted 1 in 25 in Rabbit Blocking Buffer (final concentration 0.27mg/ml) for a further one hour at 4°C. Finally, cells were washed three times in washing buffer before addition of 500µl of fixation buffer and incubation at 4°C in the dark until visualisation by confocal microscopy.

2.9.5 Confocal microscopy and quantification of microbead capture or internalisation

Cells were visualised on a Zeiss confocal microscope using BioRad Lasersharp software. An example of the images obtained is shown in figure 5.5. Three randomly selected fields of each group were imaged at low magnification. Images were studied subsequently using Adobe Photoshop Elements software. Three internalisation parameters were determined according to the following formulae:

$$\textbf{\% Cells containing beads} = \frac{\text{Number of cells containing one or more beads}}{\text{Total number of cells}} \times 100$$

$$\textbf{Number of beads per cell} = \frac{\text{Total number of internalised beads}}{\text{Total number of cells}}$$

$$\textbf{\% Cell-associated beads internalised} = \frac{\text{Total number of internalised beads}}{\text{Number of external cell associated beads} + \text{Total number of internalised beads}} \times 100$$

Table 2.18 Antibodies for Microbead Internalisation Assay

Target	Conjugate	Origin	Clone	Isotype	Manufacturer	Cat. #
Primary Antibodies						
Isotype Control	PE	Rat mAb	N/A	IgG2a	eBioscience	12-4321
Isotype Control	PE	Mouse mAb	679.1Mc7	IgG1	IO Test, Beckman Coulter	IM0670
Human CD1a	PE	Mouse mAb	BL6	IgG1	IO Test, Beckman Coulter	IM1942
Human CD80	PE	Mouse mAb	MAB104	IgG1	IO Test, Beckman Coulter	IM1976
Human CD83	PE	Mouse mAb	HB15a	IgG2b	IO Test, Beckman Coulter	IM2218
Human CD209	PE	Rat mAb	eB-h209	IgG2a	eBioscience	12-2099
Human MHC I	PE	Mouse mAb	G46-2.6	IgG1	BD Pharmingen	555553
Secondary Antibodies						
Rat IgG	FITC	Rabbit	Polyclonal		Dako Cytomation	F0234
Mouse IgG	FITC	Rabbit	Polyclonal		Dako Cytomation, F0261	Mouse IgG

2.9.6 Data handling

Data handling and statistical analysis were performed using Microsoft Excel.

Chapter 3

Oxidative Stress

3.1 Introduction

3.1.1 Oxidative stress

For life in an aerobic environment, exposure to reactive oxygen species (ROS) is continuous and unavoidable. Partially reduced metabolites of oxygen are formed during electron transfer reactions. They include superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical. As the name suggests, each of these species is capable of oxidising surrounding biomolecules, with potentially damaging consequences for the organism. As a result, a homeostatic balance exists between the formation of ROS and their removal by antioxidant scavenging compounds such as superoxide dismutase, catalase, vitamins C and E, and reduced glutathione. "Oxidative stress" arises when cellular production of ROS overwhelms its antioxidant capacity. Indeed, the overall level of oxidative stress, and the response to it, has been suggested as a major determinant of eukaryotic lifespan (Golden et al., 2002).

ROS have traditionally been regarded as toxic by-products of metabolism, however it has long been known that ROS production can be used to the advantage of the host as an innate response to invading microorganisms. Phagocytic cells such as neutrophils and macrophages produce ROS via the "respiratory burst", in which an NADPH oxidase system mediates a transient 100-fold increase in oxygen consumption with the consequent production of high concentrations of ROS, and the resultant death of engulfed microorganisms. The importance of this NADPH oxidase system to innate immunity is emphasised by the observation that defects in this system result in chronic granulomatous disease in which patients typically die in childhood of severe recurrent infections.

Oxidative stress, as a consequence of the production of ROS in the respiratory burst, is therefore a hallmark of inflammation. In the case of DC, whose role in pathogen detection and antigen presentation is central to the induction of adaptive immunity, this ROS exposure is particularly relevant. Oxidative stress is likely to be a common feature of the inflammatory microenvironment precisely when DC at these sites are receiving maturation signals provided by the invading microorganisms.

Furthermore, high concentrations of ROS can act indirectly as well as directly, by modifying the structure of proteins and other macromolecules (Alderman et al., 2002), and thus changing the repertoire of antigens that DC may process and present (Al Bukhari et al., 2002). During inflammation, damage mediated by ROS occurs at the plasma membrane leading to the generation of oxidised proteins and phospholipids and alterations in membrane permeability (Zimmerman, 1995). Cellular apoptosis is therefore an obligate outcome to prevent immune

response to intracellular self-antigens. However apoptotic cells have also been implicated in the activation of autoimmunity, in particular atherosclerosis, due to the development of autoantibodies against oxidised phospholipids and low-density lipoproteins (Chang et al., 2004). Hence the response of DCs to oxidative stress, and ROS - both how they protect themselves from excess injury, and the epitopes to which they are induced to respond - has been invoked as an important aspect of the physiology of these cells (Park et al., 2002). While it is important that DC respond and induce adaptive immunity to invading pathogens, it is also critical that responses to modified self antigens are not elicited, which could result in autoimmunity.

It is becoming increasingly apparent that in addition to their antimicrobial role, ROS also perform an essential function as second messengers in intracellular signaling. Indeed, even the respiratory burst may have more of a signaling than directly microbicidal role (Reeves et al., 2002). ROS have also recently been shown to be generated by specialised plasma membrane oxidases (which resemble the classical NADPH oxidase system of phagocytes) in response to various growth factors or cytokines (Thannickal and Fanburg, 2000). Major downstream targets of ROS production, whether physiological or pathological are MAPKs. Several mechanisms have been proposed for this MAPK activation, including receptor clustering (Peus et al., 1999), protein tyrosine phosphatase inhibition (Soberman, 2003), or MAP3K activation (Tobiume et al., 2001). JNK and p38 are therefore essential proteins in the response of cells to both physiological and hyper-physiological ROS production.

3.1.2 Chapter aims

Due to this ability to respond to both intracellularly produced low level ROS, and much greater concentrations of extracellular ROS and oxidative stress, the p38 and JNK MAPK pathways are thus able to act as sensors for the level of oxidative stress at an inflammatory site. In addition to their ability to respond to ROS, MAPK pathways, and p38 in particular, play an important role in the intracellular events that are associated with DC maturation (Bunyard et al., 2003; Xie et al., 2003). Therefore activation of p38 by ROS present at inflammatory sites might be predicted to initiate DC maturation, or modulate DC maturation induced by strong stimuli such as LPS. Less is known about the role of JNK in DC and the functional consequences of JNK activation have not been studied extensively, primarily due to the absence of selective inhibitors. We therefore wished to examine the ability of ROS to activate MAPKs in DC and to investigate the effect of this activation on DC function.

3.2 Results

3.2.1 Characterisation of dendritic cells

The purity of the DC population used for these studies was confirmed by flow cytometry. As figure 3.1a shows, the purified cells that were prepared from peripheral blood contained <1% of contaminating CD3- or CD19-positive cells (T or B cells).

Analysis of characteristic DC surface markers confirmed the identity of the DC differentiated from monocytic precursors. The DC expressed low levels of CD14 and CD25, which are both present at significantly higher levels on the monocytic precursors. The DC also expressed high levels of the myeloid marker CD13, and the characteristic DC surface proteins, CD11c, CD1a, and CD36 (figure 3.1b).

We next examined the ability of these purified DC to respond to strong DC maturation stimuli. As shown in Figure 3.2a, following 18-hour incubation with 100ng/ml LPS, the DC significantly upregulated the expression of MHC class I and II molecules, costimulatory molecules CD80 and CD86, the adhesion molecule CD54, and a surface marker present on mature DC, CD83.

Another characteristic of the maturation process is the striking ability of DC to undergo morphological alteration. Immature DC are mostly rounded cells in tissue culture, and upon overnight stimulation, the DC extend long dendritic processes. We therefore examined the ability of these purified DC to undergo morphological change upon incubation with 100ng/ml LPS. As figure 3.2b demonstrates, maturation of the DC was accompanied by extension of long dendritic processes.

Overall, we can therefore be confident that the cells used in these studies are characteristic of immature DC, and demonstrate a high level of purity.

3.2.2 Effects of H_2O_2 on DC phenotypic maturation

The ability of DC to mature in response to incubation with H_2O_2 and the TLR4 ligand LPS was followed, using the same flow cytometry approach. As expected, LPS (100ng/ml) induced up-regulation of the three surface markers examined, CD54, CD86 and HLA-DR (figure 3.3). In contrast, H_2O_2 over a range of concentrations failed to up-regulate any of the markers examined relative to unstimulated cells (figure 3.3). Incubation with 1mM H_2O_2 resulted in significant reduction in mean forward scatter, consistent with the induction of apoptosis

(data not shown). The phenotype data obtained with this concentration was therefore not analysed further.

To examine the ability of H_2O_2 to modulate the response of DC to LPS, the cells were incubated with various concentrations of the two stimuli and examined for CD86 expression, as this was one of the most robust and sensitive markers of DC maturation (figure 3.4a). There was no evidence that H_2O_2 enhanced the effects of LPS at any combination of stimuli tested, either in terms of increased sensitivity of the DC to LPS, or by increased expression of CD86 above the level induced by LPS alone. On the contrary, levels of CD86 appeared to decrease in the presence of H_2O_2. Examination of the flow cytometry plots indicated that this decrease was accompanied by a progressive decrease in cell size (figure 3.4b), indicated by a reduction in forward scatter (FSC-H), suggestive of apoptosis.

For each treatment shown in figure 3.4a, the number of gated cells (those with the defined parameters of control DC) as a percentage of the total was calculated (figure 3.4c). As the concentration of LPS or H_2O_2 is increased, the percentage of gated cells is reduced, and there is clearly a correlation between the degree of stimulation and the decreased cell size. The level of apoptosis in the DC cultures was therefore investigated in more detail.

3.2.3 Effects of H_2O_2 and LPS on DC apoptosis

Apoptosis was assessed both by measuring DNA fragmentation seen as a sub-G_0 peak on flow cytometric analysis of DNA content using PI staining (figures 3.5a and b), and by using a combination of surface annexin V and PI staining (figures 3.5c and d).

Analysis of DNA content revealed that culture of DC in the presence of 300μM H_2O_2 induced some apoptosis (figure 3.5a) with fewer cells in G_0 (figure 3.5b), consistent with the shift in Forward Scatter observed by flow cytometry. Culture of DC in the presence of LPS did not induce a significant increase in apoptosis.

Unexpectedly, however, the combination of LPS (100ng/ml) and H_2O_2 (300μM) induced apoptosis in a large proportion of the cells (figures 3.5a and b). Thus LPS and H_2O_2 appeared to show synergistic ability to activate the apoptotic pathway in DC.

The results of annexin V/ PI staining were very similar. There are fewer PI/ annexin V double negative cells in the H_2O_2 (300μM) (lower left quadrants, figure 3.5c) than in the controls; LPS

alone caused a small decrease, but the most striking findings were seen when the two modulating agents were used together.

3.2.4 Effects of H_2O_2 and LPS on p38 & JNK MAPK activation in DC

To investigate the activity of signaling pathways underlying these changes in DC maturation and survival, DC were exposed either to various concentrations of H_2O_2, or LPS, or both together, and phosphorylation of p38 and JNK was analysed by Western blot.

The level of phosphorylated p38 increases in proportion to H_2O_2 concentration in a time-dependent manner (figure 3.6a). Stimulation with 0.1mM H_2O_2 for 15 mins induced strong but transient p38 activation, while 1mM H_2O_2 induced a similar but sustained level of p38 phosphorylation. 0.1mM H_2O_2 induced low-level transient JNK activation, while 1mM H_2O_2 induced stronger and more sustained JNK phosphorylation, with some phosphorylated JNK still detectable after 4 hours of stimulation. Treatment with a hyperphysiological concentration of 10mM H_2O_2 induced cell necrosis, reflected by the reduced level of total JNK protein at 4hrs.

Both p38 and JNK were also phosphorylated in response to LPS (figures 3.6b and c). Densitometry showed that p38 was ten-fold more sensitive to activation by LPS compared to JNK (figure 3.6c).

Signal pathway responses in DC after exposure to a combination of H_2O_2 and LPS are shown in figure 3.7. Neither H_2O_2 nor LPS altered the levels of total p38 and total $p54^{JNK}$. The presence of H_2O_2 did not consistently enhance LPS-induced p38 phosphorylation (figure 3.7b). LPS (100ng/ml) and H_2O_2 (0.1mM) synergised, however, in the induction of JNK phosphorylation (figure 3.7c). This experiment was performed three times. The mean fold activation of JNK due to H_2O_2 alone was 10, for LPS alone 3.3, and for the combination 19.6.

As a stimulus, hydrogen peroxide yielded qualitatively consistent, but quantitatively inconsistent responses. This is possibly due to the high reactivity and short half-life of hydrogen peroxide resulting in slightly different effective concentrations, in combination with donor-specific variations in susceptibility to oxidative stress. Experiments were therefore performed at least three times, and, where indicated, 'representative' results, reflecting common trends are shown.

The dichotomy between the ability of hydrogen peroxide to induce p38 phosphorylation and its failure to induce DC maturation was unexpected. As the NF-κB pathway has also been

shown to be essential for DC maturation by LPS, and is activated in many cell types by H_2O_2, this pathway was also analysed. The basis for these experiments was that, prior to activation, NF-κB is sequestered in the cytoplasm by an inhibitory protein, IκB; but that upon activation, this protein is phosphorylated and then immediately degraded, allowing NF-κB to translocate to the nucleus and initiate transcription (Karin and Ben Neriah, 2000) (figure 1.4). Therefore using an anti-IκBα antibody to determine the level of inhibitory protein present, the degree of NF-κB inhibition was examined. Only LPS, but not H_2O_2, induced IκBα degradation, and hence NF-κB translocation (figure 3.7a).

3.2.5 Correlation between DC apoptosis and JNK phosphorylation

Given the reported role of JNK in pro-apoptotic signaling in other cell types (Chen and Tan, 2000), and the observations that in DC, H_2O_2 and LPS synergised in the induction of apoptosis (figure 3.5) and JNK activation (figure 3.7), we decided to further analyse the role of JNK in DC apoptosis.

DC were treated with various concentrations of H_2O_2 and then examined in parallel for the level of JNK activation and the degree of induced cell death. The level of H_2O_2-induced JNK phosphorylation, expressed as the ratio of phosphorylated- to total-JNK by Western blot and band densitometry is shown in figure 3.8a. The degree of H_2O_2-induced cell death was studied by incubating DC with H_2O_2 for one hour before change of medium and overnight incubation. Cell viability was then assessed by total DNA content (figure 3.8b), annexin V/PI staining (figure 3.8c) and MTT reduction (figure 3.8d). The degree of JNK activation (figure 3.8a) correlates closely with DC death measured by the size of the sub-G_0 population (figure 3.8b), and by annexin V/ PI positivity (figure 3.8c), and this correlation is shown in fig 3.8e. The cells treated with 10mM H_2O_2 were necrotic by light microscopy and trypan blue exclusion (data not shown), and this is consistent with the data shown in figure 3.8e because the percentage cell death measured by annexin V/ PI (81.15%) includes necrotic cells, whereas the Sub-G_0 quantification (21.2%) gives a measure of the apoptotic population only.

3.2.6 Inhibition of p38 & JNK MAPK

The involvement of the JNK pathway in DC apoptosis was examined further using two selective low molecular weight JNK inhibitors, CEP11004 (CEP), an inhibitor of Mixed Lineage Kinase (MLK), a MAP3K upstream of JNK (Murakata et al., 2002), and SP600125 (SP), an ATP-binding site analogue competitive inhibitor of JNK (Bennett et al., 2001). The pharmacological activity of these two inhibitors in DC was first confirmed by Western blot

(figures 3.9a and b). CEP inhibited H_2O_2-induced JNK activation in a dose-dependent manner (figure 3.9a). No JNK phosphorylation is detectable after pre-treatment with 1μM CEP, suggesting that H_2O_2 induces JNK activation in DC exclusively via MLK activation. In contrast, SP is a competitive and reversible inhibitor of JNK itself, rather than an upstream kinase, and hence its efficacy was measured as the ability of the inhibitor to prevent phosphorylation of c-jun, a substrate of JNK. As predicted, activation of c-jun is inhibited in a dose dependent way by SP (figure 3.9b). At higher concentrations (10μM), SP did in fact also inhibit JNK phosphorylation, indicating that at this concentration, inhibition of additional kinases occurs. Indeed, in initial analysis of the inhibitor, the manufacturers determined that SP inhibits the JNK-specific MAP2K MKK7 with an IC50 value approximately ten times greater than that for JNK inhibition, which supports the observations of this study (Bennett et al., 2001). However, at lower concentrations, there was significant inhibition of c-jun in the absence of JNK inhibition (figure 3.9b). Such non-specific inhibition is a limitation of inhibitor studies because the effects of the inhibitors on every protein kinase cannot be determined.

Finally, as expected, figure 3.9c shows that the control p38 inhibitor, SB203580 (SB), did not have a strong inhibitive effect on either JNK or c-jun phosphorylation at any concentration used (up to 10μM). Also in line with expectations, neither the p38- (SB), nor JNK-specific (SP) inhibitors prevented phosphorylation of p38. However, inhibition of MLK by CEP did prevent H_2O_2-induced p38 phosphorylation, suggesting that this protein lies upstream of both p38 and JNK in the H_2O_2 response pathway. This p38 and JNK activatory role of MLK in DC is examined in more detail in Chapter 4.

3.2.7 Effects of MAPK inhibitors on apoptosis

To determine the role of JNK in mediating H_2O_2-induced DC apoptosis, H_2O_2 was added overnight to cells pre-treated with CEP, SP and SB inhibitors. Cell viability was determined by measuring annexin V/PI (figure 3.10a), DNA content (figure 3.10b) and capacity to reduce MTT (figure 3.10c). Both JNK inhibitors increased the proportion of viable cells in all three assays, providing partial protection against H_2O_2-induced cell death (figure 3.10d). In contrast, the p38 inhibitor SB was much less effective in this regard. The percentage protection facilitated by each inhibitor was calculated and is shown in figure 3.10d. Neither the inhibitors themselves, nor their solvent DMSO affected cell viability under these conditions (figure 3.10b).

3.2.8 Effects of p38 and JNK inhibition on DC maturation

The role of JNK in LPS-induced DC maturation has not previously been examined, and therefore the ability of the JNK inhibitor SP on LPS-induced CD86 upregulation was studied. As a positive control, the p38 inhibitor SB was used. As expected from previous reports, p38 inhibition completely blocked the ability of LPS to induce CD86 up-regulation (representative histograms (figure 3.11a) and mean fluorescence intensity of three experiments (figure 3.11b)) Partial inhibition was seen at 3μM, and total inhibition at 10μM, as a result 10μM was used in all further studies. In contrast the JNK inhibitor SP had no effect on phenotype (figure 3.11a and b), suggesting that the JNK MAPK pathway is not involved in modulating DC phenotype.

3.2.9 Effects of H_2O_2 on protein tyrosine phosphorylation

The mechanism of JNK activation by H_2O_2 has not been studied in DC. In other cell types the MAP3K protein Apoptosis Signal Regulating Kinase-1 (ASK-1) has been implicated in this process (Tobiume et al., 2001), but this is not consistent with the ability of CEP to inhibit H_2O_2-induced JNK activation and apoptosis as shown in figure 3.9a. The effects of the CEP inhibitor suggest that MLK, rather than ASK-1, is the main activator of JNK in DC in response to H_2O_2.

There is also an alternative proposed mechanism of H_2O_2-mediated JNK activation, via transient inhibition of protein tyrosine phosphatases (PTPs), due to reaction of H_2O_2 with the catalytic cysteine in the active site of this class of enzyme. If this were the case then phosphorylated tyrosine residues would accumulate upon H_2O_2 treatment. Physiologically, this phosphatase inhibition would need only to be very brief and hence would be difficult to observe; but hyperphysiological concentrations of H_2O_2 would induce more pronounced inhibition and accumulation of phosphorylated tyrosine residues would be observed. To test this hypothesis, DCs were treated with various concentrations of H_2O_2 and total protein tyrosine phosphorylation analysed by Western blotting.

Figure 3.12a shows that there was indeed a dose-dependent increase in the level of tyrosine phosphorylation upon treatment of DC with H_2O_2. Furthermore, figure 3.12b shows that the level of tyrosine phosphorylation rapidly diminished upon removal of H_2O_2. To further investigate the hypothesis that H_2O_2 activates MAPK in DC via inhibition of PTPs, the protein tyrosine phosphatase inhibitor sodium orthovanadate (Na_3VO_4) was used (Huyer et al., 1997). DCs were treated with different concentrations of Na_3VO_4, lysed after 15 minutes, and then analysed by Western blot. As shown in Figure 3.12c, Na_3VO_4 induces a high degree of tyrosine

phosphorylation similar to that seen in the presence of H_2O_2. The membranes were stripped and re-probed for phosphorylated JNK and p38. Na_3VO_4 induced phosphorylation of both JNK and p38 (figure 3.12c). Furthermore, in striking resemblance to H_2O_2 (and LPS), p38 was phosphorylated at a lower concentration (10μM) than JNK (100μM).

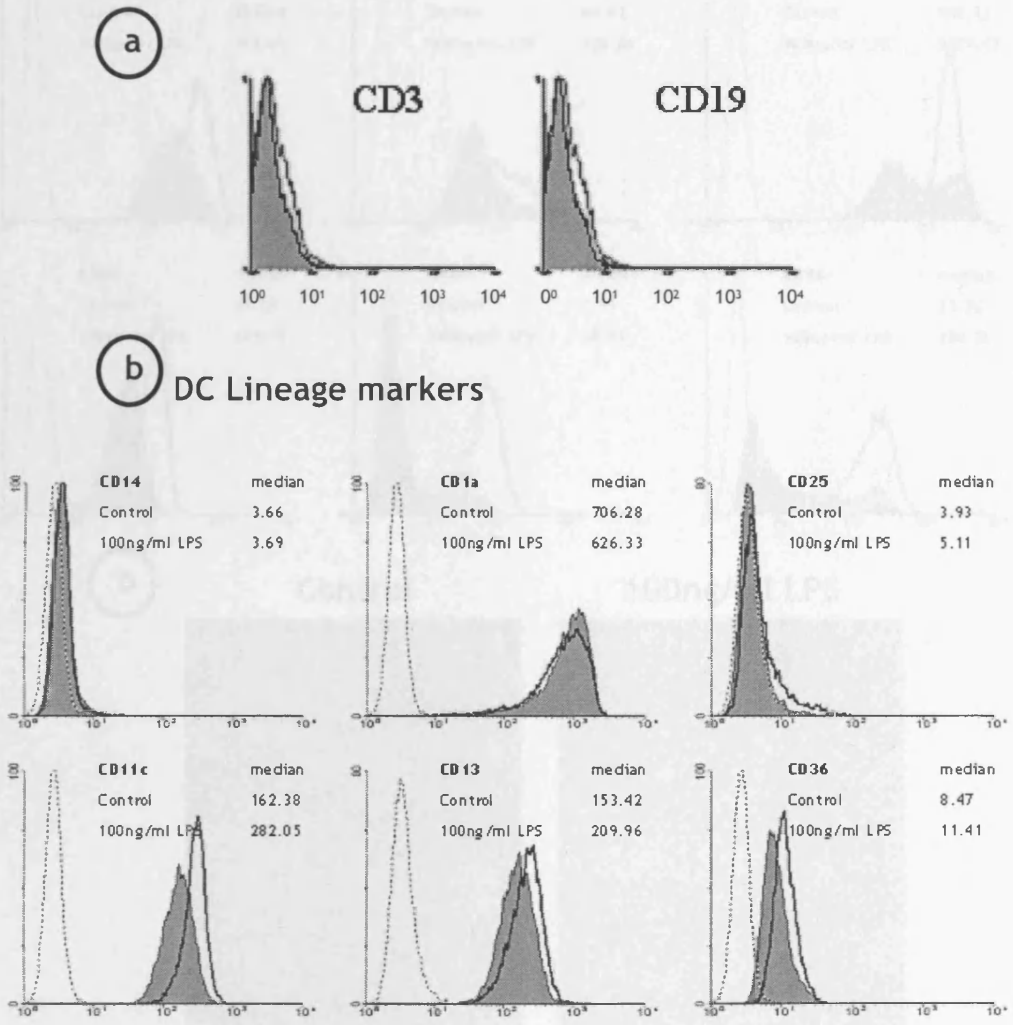


Figure 3.1. Analysis of purity

(a) The DC population differentiated from blood monocyte precursors was examined for the presence of contaminating cells. Expression of surface marker proteins CD3 and CD19, characteristic of T and B cells, was analysed by flow cytometry. Unfilled histograms - Isotype control (see M&M). Filled histograms - test antibody. (b) Immature DC were incubated with (unfilled histograms) or without (shaded histograms) 100ng/ml LPS for 18 hours. Expression of surface marker proteins was then analysed by flow cytometry. Dashed histograms - Isotype control.

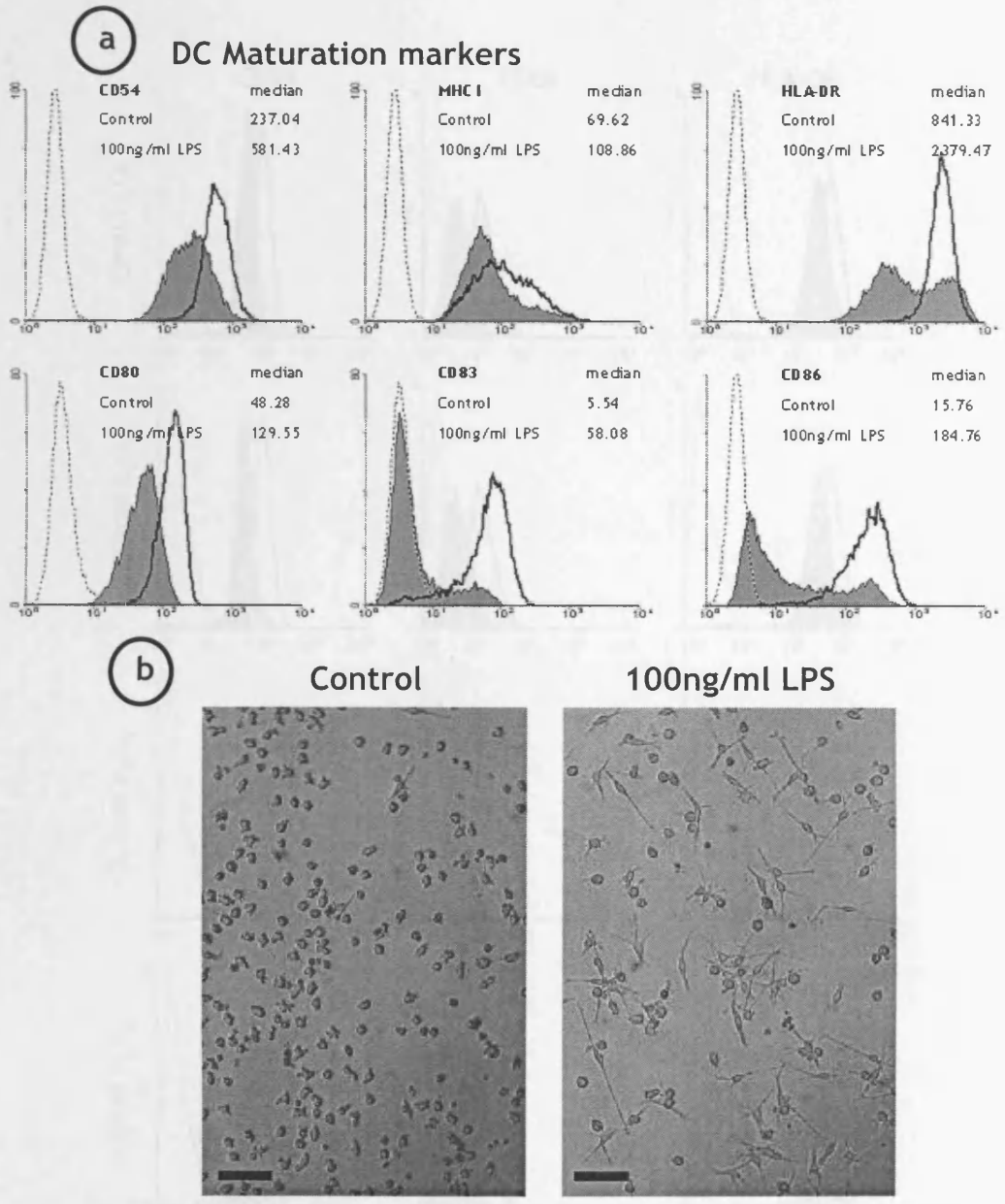


Figure 3.2. Characterisation of DC Maturation

(a) Immature DC were incubated with (unfilled histograms) or without (shaded histograms) 100ng/ml LPS for 18 hours. Expression of surface marker proteins was then analysed by flow cytometry. Dashed histograms - Isotype control. (b) Morphological characterisation of immature DC in tissue culture analysed by light microscopy. Left panel - unstimulated. Right panel - incubation with 100ng/ml LPS for 18 hours. Scale bar represents 50μm.

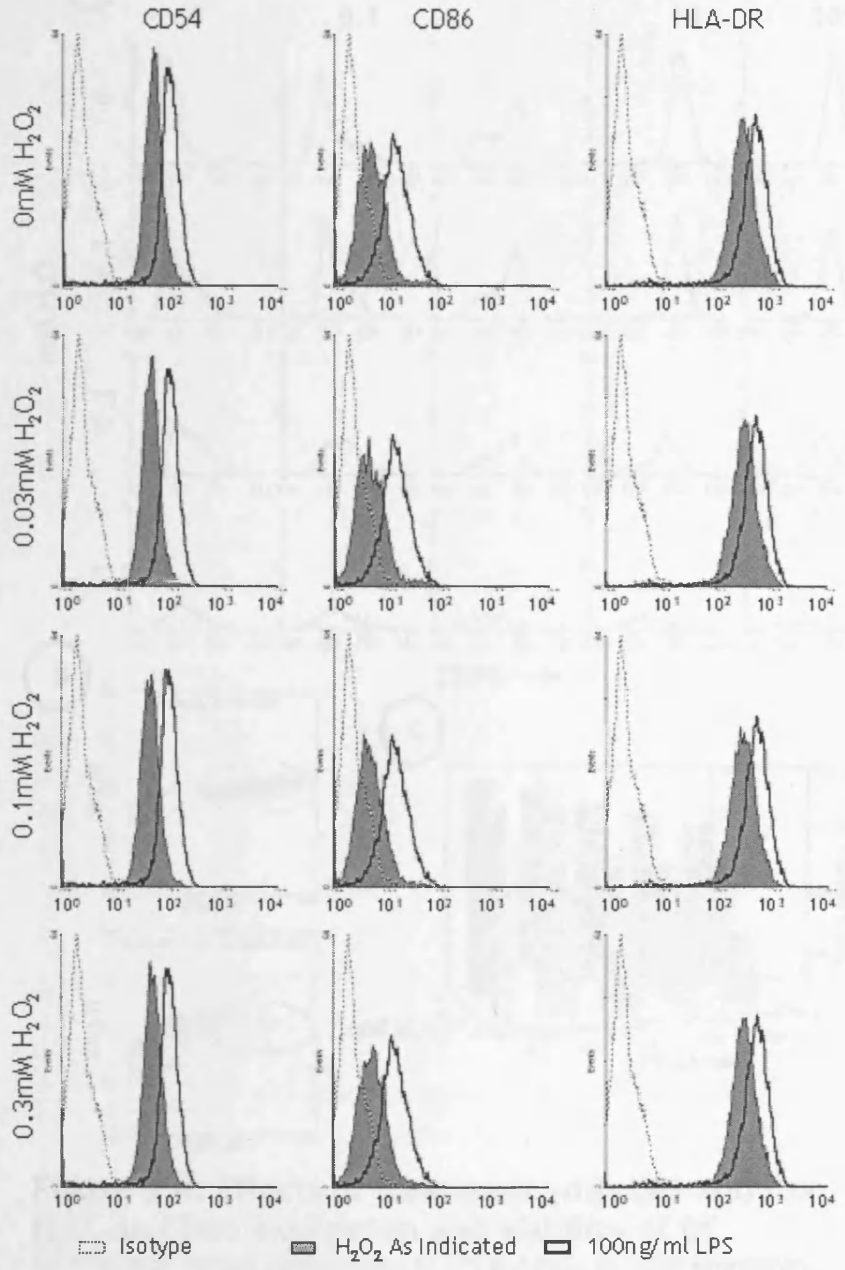


Figure 3.3. Effects of LPS & H_2O_2 on DC phenotype

Immature DC were incubated with or without 100ng/ml LPS or various concentrations of H_2O_2 as indicated for 18 hours. Expression of surface marker proteins was then analysed by flow cytometry. One representative of three experiments is shown.

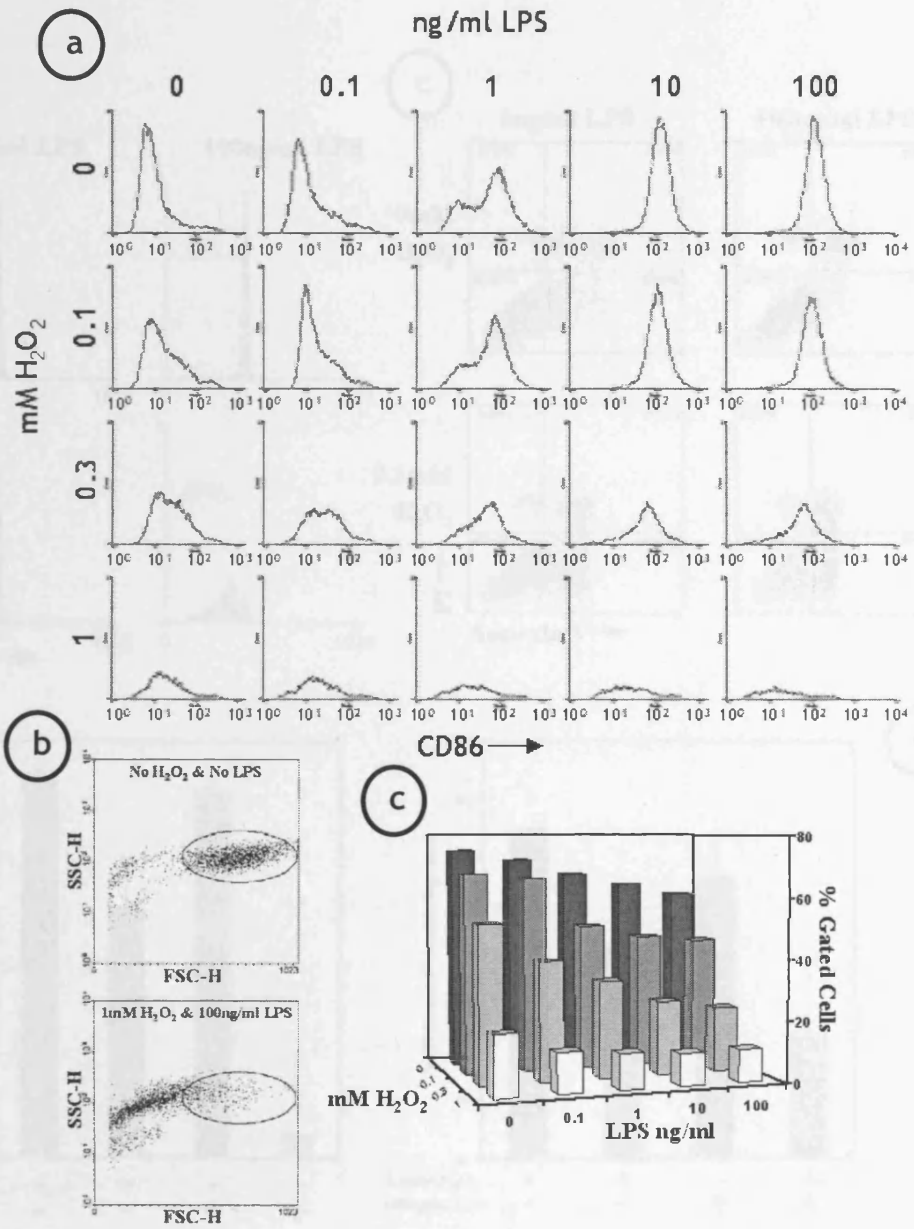


Figure 3.4. Effects of treatment with LPS and / or H_2O_2 on CD86 expression and viability of DC

(a) Effects of various combinations of LPS and H_2O_2 on CD86 expression. Immature DC were treated with various concentrations of LPS and H_2O_2 as indicated for 18 hours and then analysed for CD86 expression by flow cytometry. Representative of three independent experiments. (b) A representative forward scatter/ side scatter analysis of cell populations characteristic of healthy and apoptotic DC showing the decrease in the percentage of gated cells associated with cell death. (c) DC were treated with or without LPS and/ or H_2O_2 for 18 hours and then analysed by flow cytometry as in panel (b). The results show the percentage of 20,000 counted events occurring within the size and granularity gate defined for untreated DC. Representative of three independent experiments.

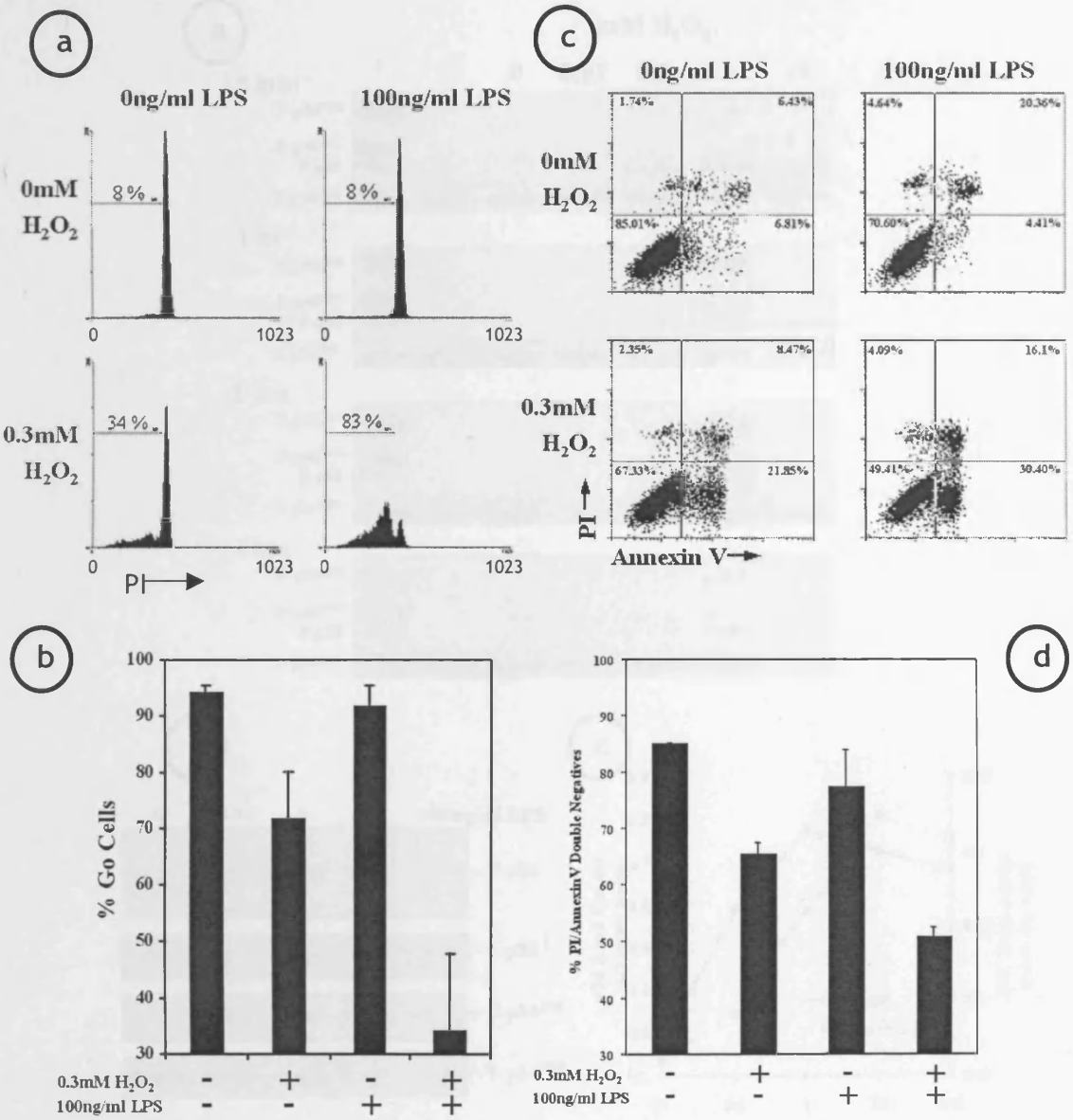


Figure 3.5. Analysis of DC viability upon treatment with LPS and / or H_2O_2

(a) DC were incubated overnight with medium only, or 100ng/ml LPS and/ or 0.3mM H_2O_2 prior to analysis of DNA content by propidium iodide staining and flow cytometry. Live cells show a single peak of DNA corresponding to cells in G_0/G_1 stage of the cell cycle. During apoptosis, DNA is degraded and observed as a sub-G_0 peak. One representative experiment of 4 is shown. (b) Bar chart shows mean percentage of DC in G_0 gate of four independent experiments. Error bars represent standard error of the mean (SEM). (c) As for panel (a) but cell viability was assessed by Annexin V/PI staining. (d) Bar chart showing mean viable cells (lower left quadrant) as measured in (c) from three independent experiments. Error bars represent standard error of the mean.

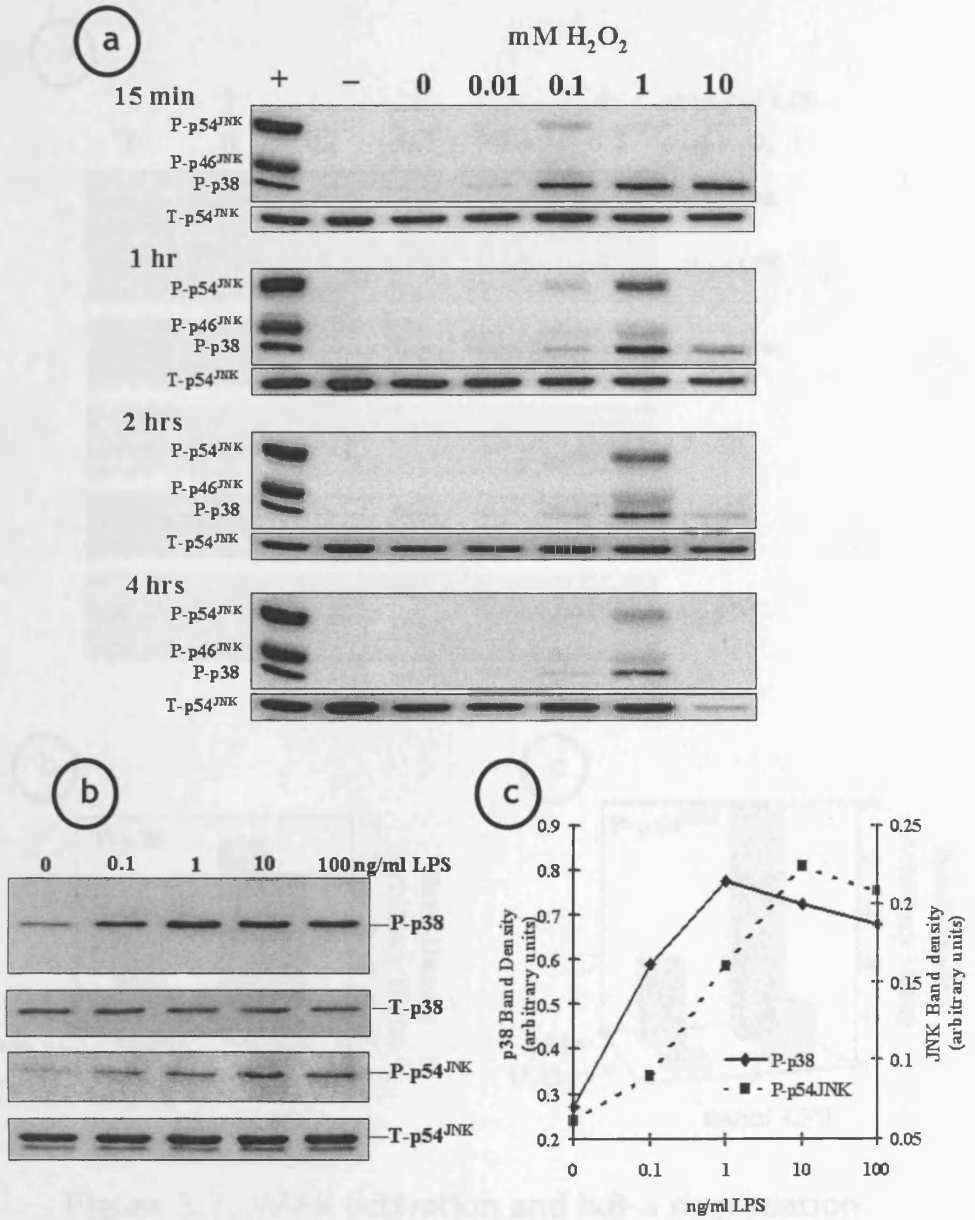


Figure 3.6. MAPK activation in DC treated with LPS or H_2O_2

(a) DC were treated with H_2O_2 at the concentrations shown. At various times post-stimulation cells were harvested, lysed and analysed by Western blot for the total and phosphorylated forms of the MAPK as indicated. Positive and negative control lysates were prepared from 293T cells stimulated or not with UV light for 60 seconds. Representative of three independent experiments. (b) DC were treated with various concentrations of LPS for 60 minutes, lysed and analysed by Western blot for total and phosphorylated forms of MAPK as indicated. (c) Density of bands on autoradiographs from (b) analysed by densitometry. Representative of three independent experiments.

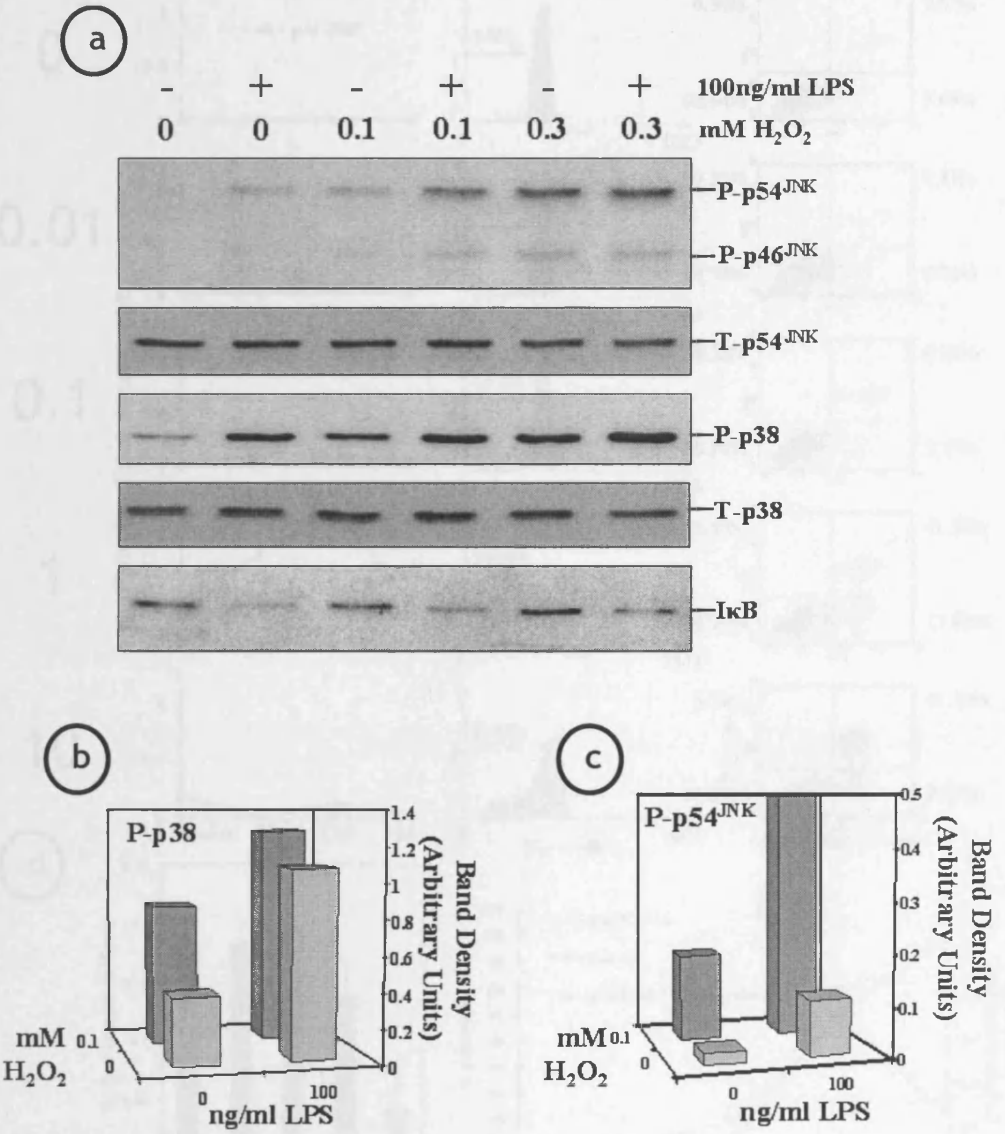


Figure 3.7. MAPK activation and IκB-α degradation in DC treated with LPS and H_2O_2

Response of DC to dual stimulation by LPS and H_2O_2. (a) DC were treated with various concentrations of LPS and H_2O_2 for 60 minutes, lysed and analysed by Western blot for total and phosphorylated forms of MAPK or total IκB-α as indicated. (b) and (c) The density of selected bands from the autoradiographs analysed by densitometry. Representative of three independent experiments.

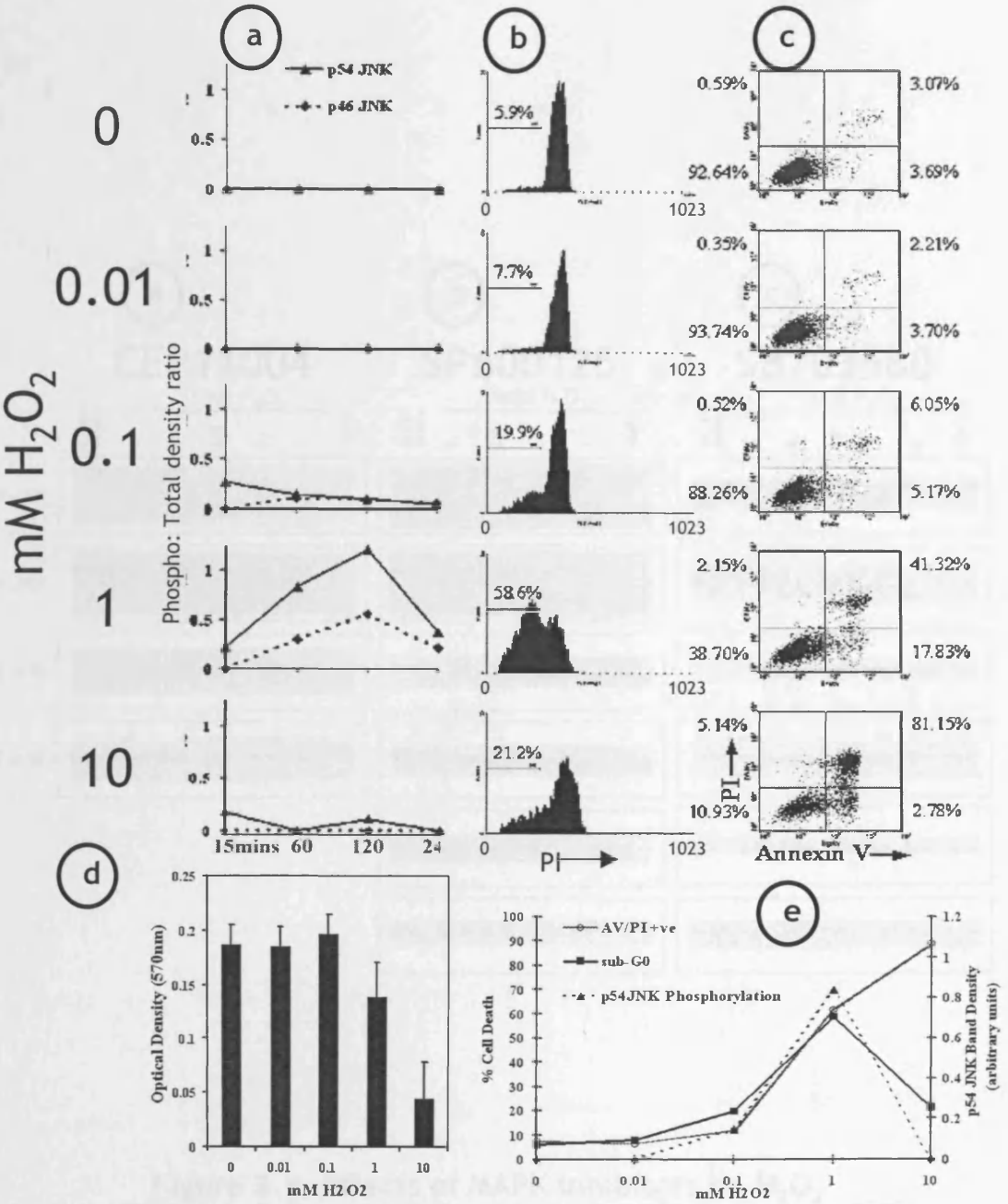


Figure 3.8. Correlation between apoptosis and JNK activation induced by H_2O_2

(a) DC were treated with H_2O_2 at the final concentrations shown. At various times post-stimulation cells were removed, lysed and analysed by Western blot. The density of phospho- and total-JNK p54 bands on the autoradiographs were analysed by densitometry. Phospho: Total density ratios are shown. Representative of three independent experiments. (b) DC were treated for one hour with various concentrations of H_2O_2 as shown. Cells were then washed and incubated in fresh medium overnight before analysis of total DNA content by propidium iodide staining and flow cytometry. Representative of three independent experiments is shown. (c) as for panel (b) except cell viability was assessed by annexin V/ propidium iodide staining and flow cytometry. Representative of three independent experiments is shown. (d) as for panel (b) except cell viability was assessed by ability to reduce MTT. Mean of three independent experiments is shown. (e) Correlation between JNK activation and DC apoptosis. JNK activation is indicated by the ratio of Phospho: Total JNK p54 western blot band density following one-hour treatment with H_2O_2. The degree of apoptosis indicated is the % sub-G_0 DC population as shown in (b), or the % PI positive and/or Annexin V positive DC as shown in (c).

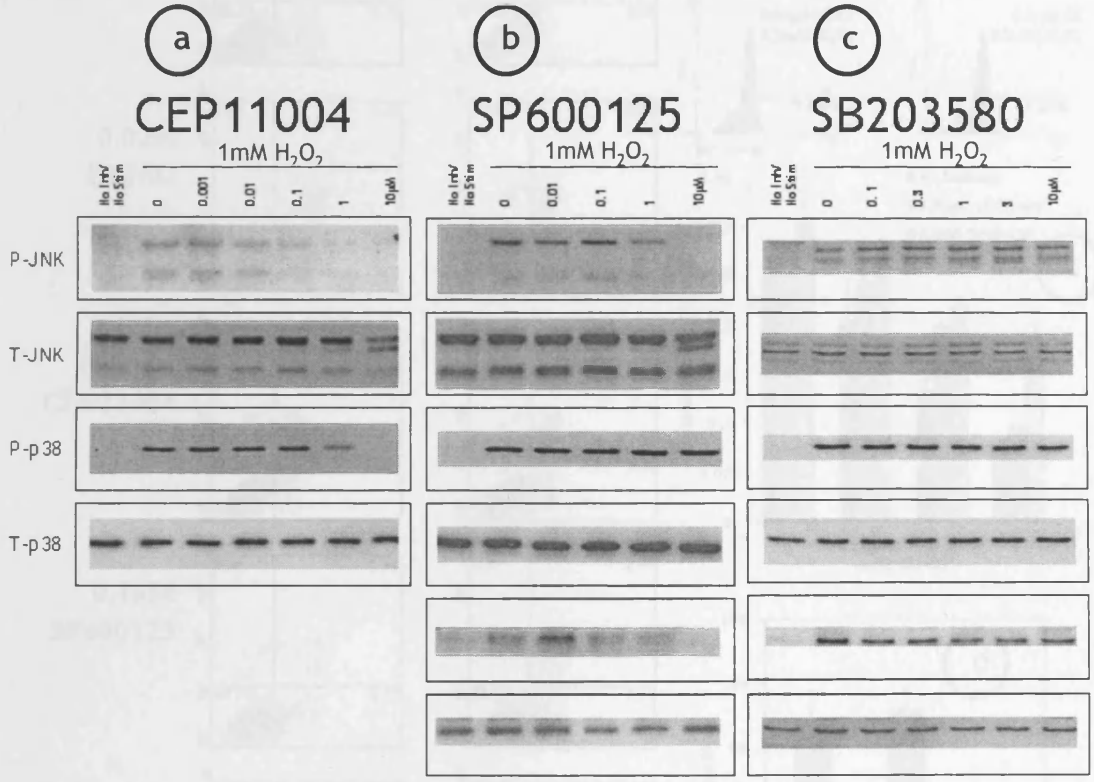


Figure 3.9. Effects of MAPK inhibitors on H_2O_2-induced JNK, p38, and c-jun activation

DC were incubated overnight with CEP11004 (a), SP600125 (b), or SB2032580 (c) inhibitor at the concentrations indicated. 1mM H_2O_2 was then added for 60 minutes prior to lysis and examination by Western blot for total or phosphorylated forms of JNK, p38 or c-jun proteins as indicated.

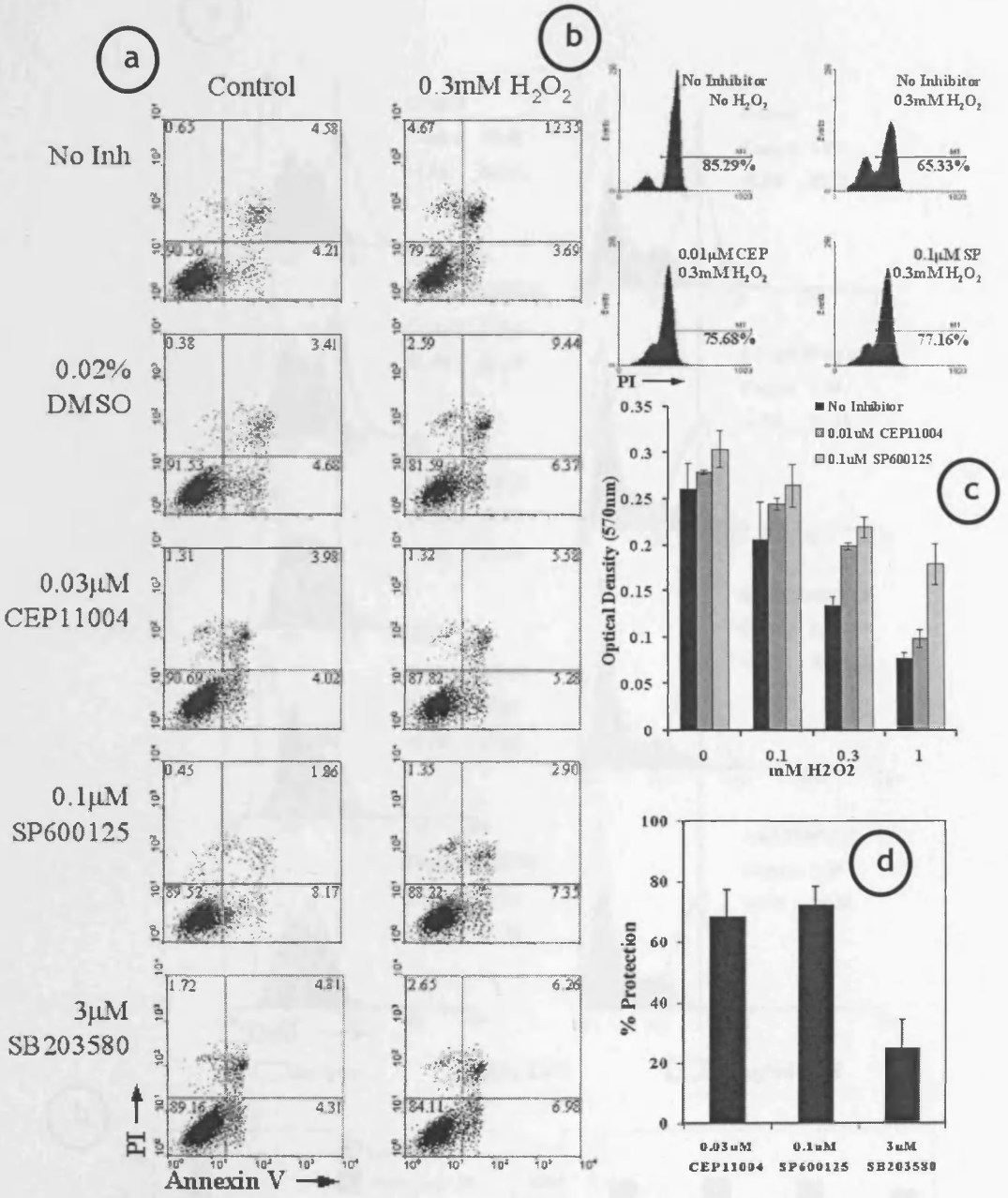


Figure 3.10. Effects of MAPK inhibitors on H_2O_2-induced DC apoptosis

DC were incubated overnight with CEP11004, SP600125 or SB203580 inhibitor prior to addition of H_2O_2 and incubation for a further 18 hours. Cell viability was then assessed by total DNA content by Annexin V/ propidium iodide staining and flow cytometry (a), by propidium iodide staining and flow cytometry (b), and by MTT reduction (c). (a) and (b) show results from one representative experiment of three, while (c) shows the mean of three experiments. (d) Mean percentage protection calculated from Annexin V/PI apoptosis measurements using the formula $[(\%LL_{Inh\ \&\ H2O2\ treated} - \%LL_{H2O2\ treated})/ (\%LL_{Untreated} - \%LL_{H2O2\ treated}) \times 100]$ (where LL represents the % DC in the lower left quadrant) from three experiments at optimal inhibitor concentrations.

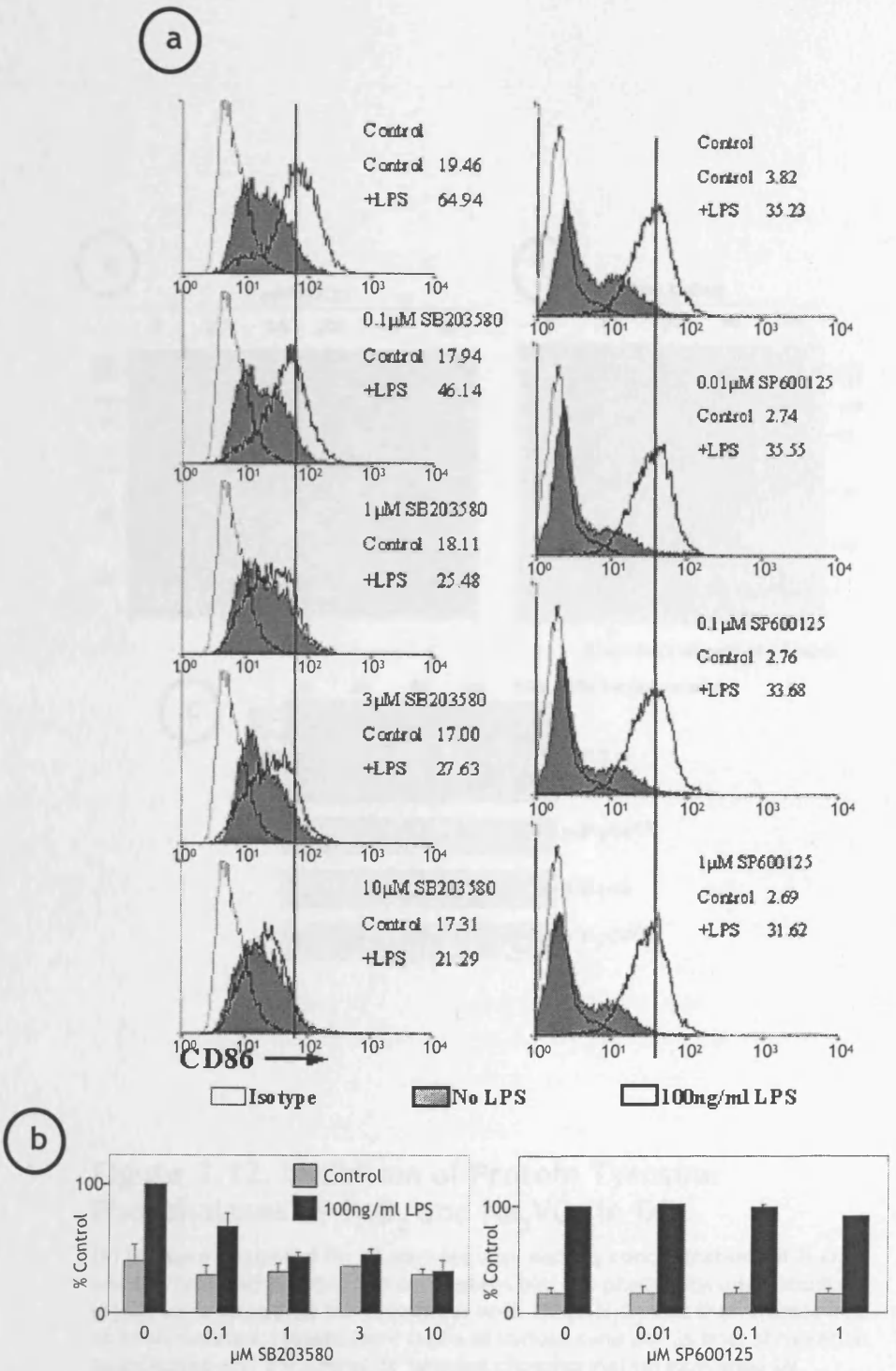


Figure 3.11. Effects of MAPK inhibitors on LPS-induced CD86 up-regulation

DC were incubated overnight with SB203580 or SP600125 inhibitor prior to addition of LPS (where indicated) and incubated for a further 18 hours. Expression of surface marker proteins was then analysed by flow cytometry. One representative (a), and mean median fluorescent intensities (b), of three independent experiments are shown.

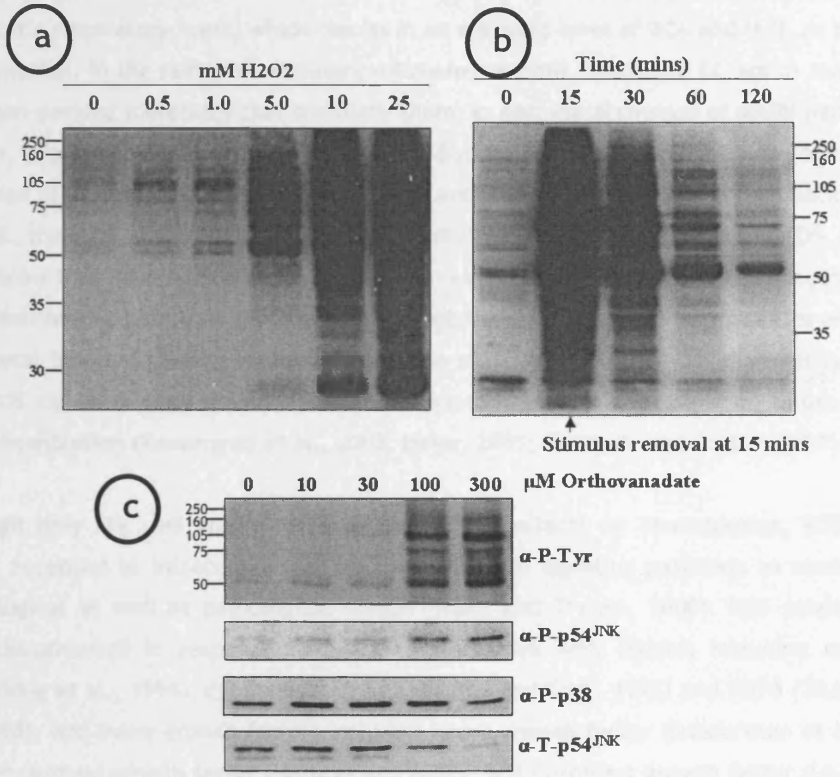


Figure 3.12. Inhibition of Protein Tyrosine Phosphatases by H_2O_2 and Na_3VO_4 in DC

(a) DC were incubated for 15 minutes with various concentrations of H_2O_2 prior to lysis and examination by Western blot for phosphotyrosine residues. (b) DC were incubated for 15 minutes with 10mM H_2O_2 and then transferred to fresh medium. Lysates were made at various time points post stimulation as indicated and the degree of tyrosine phosphorylation examined by Western blot. (c) DC were treated for 15 minutes with various concentrations of Na_3VO_4 as shown prior to lysis and examination by Western blot for phosphotyrosine, total or phosphorylated forms of MAPK as indicated. All blots representative of three independent experiments.

3.3 Discussion

An important mechanism of innate immune response to invading microorganisms is the phagocytic respiratory burst, which results in an elevated level of ROS and H_2O_2 at the site of inflammation. In the same inflammatory microenvironment, immature DC act as sentinels for pathogen-derived molecules that stimulate them, in part via activation of MAPK pathways, to mature, migrate to the draining lymph node and stimulate an adaptive immune response. This induction of DC maturation will therefore frequently occur at the site of inflammation, and as a result, these DC are likely to be exposed to very high local concentrations of ROS, as well as to proteins that have been altered by oxidation (Alderman et al., 2002). The susceptibility of DC to deleterious effects of these products is not the same as that of lymphocytes either from peripheral blood, or from lymphoid tissue. The situation is further complicated by the fact that ROS can have both activating and pro-apoptotic effects depending on target cell type and concentration (Kantengwa et al., 2003; Meier, 2001; Carmody and Cotter, 2001).

Although they are well known for their destructive effects on biomolecules, ROS are now widely accepted as intracellular second messengers in signaling pathways as modulators of physiological as well as pathological stimuli (Allen and Tresini, 2000). ROS production has been documented in response to cellular stimulation with ligands including angiotensin (Griendling et al., 1994), cytokines such as TNFα (Lo and Cruz, 1995) and TGFβ (Thannickal et al., 1998), and many growth factors including nerve growth factor (Sundaresan et al., 1995), platelet-derived growth factor (Meng et al., 2002), and fibroblast growth factor (Lo and Cruz, 1995). The precise mechanism of ROS signaling is incompletely understood, however, although ROS have been shown to enhance the function of various effector pathways including NF-κB (Schreck et al., 1991), MAPKs (Wang et al., 1998), tyrosine kinases (Hardwick and Sefton, 1995), and PKC (Whisler et al., 1994).

Exposure to H_2O_2 has been implicated in signaling via MAPKs in a variety of cell types previously. In the immune system there has been detailed analysis of T cell responses using cell lines, and T cell receptor (CD3/ CD28) stimulation (Los et al., 1995; Hehner et al., 2000; Kwon et al., 2003). In this study we have investigated the effect of H_2O_2 on MAPKs in DC. We have also compared this with the response of DC not only to other maturation stimuli alone, but also with maturation stimuli and H_2O_2 in a synergistic fashion, thereby attempting to mimic the in vivo dual stimulation that is likely to be seen in inflammation. Our findings confirm that both H_2O_2 and LPS activate the p38 MAPK pathway in DC. Whilst with LPS this coincides with DC maturation, no phenotypic changes were seen with H_2O_2 (figures 3.3 and 3.4), in agreement with a recent study (Kantengwa et al., 2003).

This uncoupling of the DC surface phenotype and p38 activation in DC is unexpected. As discussed in section 1.2.2, many previous studies have shown that p38 is part of the pathway leading to DC maturation in response to a variety of stimuli (Puig-Kroger et al., 2001; Ardeshna et al., 2000; Hacker et al., 1998). Furthermore, activation of p38 in DC by contact sensitizers has been shown to be mediated via generation of intracellular ROS (Mizuashi et al., 2005b; Bruchhausen et al., 2003; Mizuashi et al., 2005a), and ROS production in DC has also been demonstrated during antigen presentation (Matsue et al., 2003). Other studies have shown that H_2O_2 can induce (or amplify) DC cytokine synthesis (Verhasselt et al., 1998), where the MAPK pathway may be an upstream component. Indeed, previous investigations have suggested a role for ROS in DC phenotypic maturation (Rutault et al., 1999; Basu et al., 2000). This latter apparent contradiction can perhaps be reconciled by the fact that in contrast to these previous studies data presented here was obtained using a very highly purified population of DC based upon an additional immunomagnetic negative selection step on day 4 of culture (Alderman et al., 2002). DC prepared without this additional purification step do show a small upregulation of CD86 and MHC class II molecules in response to H_2O_2 (data not shown). Therefore we speculate that H_2O_2 induces DC maturation indirectly, and that this may be via effects on other cells and/ or their protein degradation products within the culture. For example, necrosis of lymphocytes in a surrounding microenvironment - and subsequent release of heat shock proteins may be a significant variable extrinsic to the DC, which has an influence on phenotype (Basu et al., 2000; Vabulas et al., 2002).

Our results confirm that activation of p38, while necessary, is not sufficient to stimulate DC maturation, indicating that alternative pathways are required. The activation of the NF-κB pathway is an obvious candidate (Rescigno et al., 1998; Yoshimura et al., 2001). NF-kB activation has indeed previously been shown to be essential for LPS-induced cytokine production (Bondeson et al., 1999). Accordingly, we found that H2O2 was incapable of inducing IκBα degradation and thus activation of NF-κB (figure 3.7a).

A dual p38/ NF-κB requirement is consistent with a recent study demonstrating that activation of p38 results in the modulation of chromatin structure and enhanced accessibility of NF-κB to the relevant elements in the promoters of cytokine genes and presumably other genes involved in DC maturation (Saccani et al., 2002). This model suggests that p38 is the coarse control while NF-kB controls the finer elements of the mature phenotype. H_2O_2 may therefore not activate DC despite inducing p38 phosphorylation because it does not activate NF-κB, which is known to be required for DC maturation.

This hypothesis is supported by the fact that, in contrast to LPS, H_2O_2 did not induce IκBα degradation (figure 3.7a). Whenever LPS was present in the cultures there was less IκBα; i.e. there was more phosphorylation and degradation. Furthermore, H_2O_2 did not prevent LPS-induced IκBα degradation. Both of these observations correlate with the observed effects on DC phenotype; unlike LPS, H_2O_2 did not induce upregulation of any of the markers of maturation studied (figure 3.3), and moreover H_2O_2 did not prevent LPS-induced DC phenotypic maturation (figure 3.4a).

This inability of H_2O_2 to activate NF-κB is in contrast to many previous findings in other cell types. By using metabolic inhibitors and antioxidant enzymes it has been well established that oxygen radicals are involved in NF-κB activation, and that H_2O_2 alone can activate NF-κB, although the mechanism remains unclear. ROS have even been suggested to regulate NF-κB activation in response to TLR4 ligation in neutrophils (Asehnoune et al., 2004). Indeed, a model has been suggested proposing that NF-κB is redox sensitive and that its activation is due to the production of ROS by all agonists (Schreck et al., 1991), however this obligatory requirement for ROS has since been shown to be cell- and stimulus-specific (Majumdar et al., 2002; Li and Karin, 1999; Rahman et al., 2004; Taylor et al., 2004). Alternatively, it remains a possibility that H_2O_2 does induce NF-κB activation in DC, but in the absence of IκBα degradation. Direct studies of NF-κB nuclear translocation and DNA binding capacity will be required to answer these questions.

The current molecular model whereby p38 plays a role in facilitating subsequent NF-κB-mediated transcription in DC (Saccani et al., 2002) may explain why the activation of p38 by H_2O_2 does not enhance the maturation activity of LPS. The level of phospho-p38 reaches a plateau at 1ng/ml LPS (figure 3.6b and c). However, CD86 levels at this LPS concentration are submaximal (figure 3.4), proving that although essential for the process, p38 is not the limiting factor in upregulation of this maturation marker. According to the model, p38 may not be the limiting factor in CD86 upregulation because p38 is only required to make the promoters accessible to NF-κB. Once a level of phospho-p38 is attained at which NF-κB can access DNA, an increase in phosphorylated p38 will not necessarily lead to any further increase in maturation. Presumably 1ng/ml LPS induces sufficient phospho-p38 to allow CD86 promoter accessibility, but insufficient activated NF-κB for maximum CD86 transcription. H_2O_2 does not increase CD86 levels because despite phosphorylating p38, it does not induce further NF-κB activation.

Due to the availability of the selective inhibitors, the role of p38 in controlling DC maturation is well established. At the time of undertaking the experiments presented here, however, the

role of the JNK pathway in this process had not been studied in human DC. We observed that the JNK inhibitor SP does not affect the ability of LPS to upregulate the costimulatory molecule CD86, establishing that JNK is not involved in controlling DC phenotypic maturation (figure 3.11). These findings have since been confirmed in recent published studies. In particular, a study by Nakahara *et al* examines in detail the response of DC to LPS in the presence of the SP JNK inhibitor (Nakahara et al., 2004). The results must be interpreted with care, because up to 20μM of the inhibitor was used, a concentration that we found to have non-specific effects (figure 3.9b). In fact, the authors confirm this assessment by demonstrating inhibition of JNK phosphorylation at concentrations above 1μM, in agreement with our own findings (figure 3.9b), despite the fact that inhibition of JNK itself should not prevent JNK phosphorylation. The data they present using 1μM SP is also extremely consistent with those presented here. In contrast to the control SB p38 inhibitor, they find no effect of JNK inhibition on LPS-induced upregulation of several DC maturation markers, including CD86. Expression of CD54 and CD80 demonstrate slightly reduced up-regulation in the presence of the inhibitor, suggesting a minor role for JNK in the control of the LPS-induced expression of these proteins. Unfortunately, we did not examine the effects of the inhibitor on the expression of these markers. The authors also found no effect of JNK-inhibition on the secretion of a panel of cytokines including IL12p70, IL6 and TNFα. Overall, the findings of Nakahara et al provide strong support for our conclusion that JNK has little or no role in the control of DC maturation in response to LPS.

There is also further support for such an interpretation provided by other studies. For example, it has been shown that it is possible to induce DC maturation including expression of surface and secreted proteins and T cell proliferation in the absence of JNK activation (Brittingham et al., 2005), and furthermore, the regulation of CD86 expression on dendritic cells by LIGHT has been shown to be mediated through a JNK-independent signal transduction pathway (Zou and Hu, 2005).

In contrast to the absence of synergy between H_2O_2 and LPS in terms of upregulation of DC phenotype, co-operation between H_2O_2 and LPS is clearly observed in both JNK activation (figure 3.7) and in the induction of DC apoptosis (figure 3.5). JNK activation induced by H_2O_2 alone correlates well with DC apoptosis (figure 3.8). Furthermore, CEP (figure 3.9a), and SP (figure 3.9b) both inhibit JNK and cause significant inhibition of cell death (figure 3.10). In contrast, inhibition of p38 had much less effect on H_2O_2-induced apoptosis, suggesting a limited role for p38 in this process.

In RAW macrophages, inhibition of RANK-induced JNK by SP prevents apoptosis (Bharti et al., 2004), and overexpression of a JNK kinase induced apoptosis, supporting the hypothesis that JNK regulates apoptosis in myeloid cells. Indeed, a role of JNK in stress-induced pro-apoptotic signaling is consistent with much data in other cell types (Chen and Tan, 2000), although the precise role of JNK in H_2O_2-induced apoptosis is not clear. In some cell types, the pro-apoptotic JNK response is modulated, like many JNK-dependent processes, via AP1-dependent transcription. Both SP and CEP inhibitors prevented c-jun (and hence AP1) activation by H_2O_2, indicating that this is a plausible mechanism in this case. One such protein controlled by JNK in this fashion is FasL, which is expressed on the cell surface, and mediates autocrine Fas ligation, and hence Fas-dependent apoptosis (Suhara et al., 1998). This mechanism is unlikely to apply to DC, however, since Fas engagement has been shown to induce DC maturation rather than apoptosis (Rescigno et al., 2000). Indeed, DC express high concentrations of cFLIP and this is thought to prevent caspase activation by death-domain containing surface receptors (Willems et al., 2000).

JNK-induced apoptosis may also occur via transcriptionally independent mechanisms. For example, it has recently been shown that JNK activation by TNFα causes processing of the Bcl2-related protein Bid into jBid, which then targets the mitochondria to release the apoptogenic factor smac/Diablo, which activates caspase-8 (Deng et al., 2003). JNK has also been shown to activate the caspase-9 pathway by inducing cytochrome c release from the mitochondria (Tournier et al., 2000) in response to UV radiation, although the mechanism for this is unclear.

In a recent report it has been shown that DC survival is critically dependent on the relative activities of NF-κB, which has a pro-survival function, and JNK/ AP1, which (consistent with our own findings) is pro-apoptotic (Kriehuber et al., 2005). Furthermore, these authors suggest that DC survival is enhanced by suppression of JNK by NF-κB, and this is achieved by NF-κB-dependent suppression of ROS levels, which function to activate JNK (also consistent with data presented here). According to this model, ligation of different DC surface receptors induces different relative activities of these two transcriptional activation pathways, and thus different DC longevities. Indeed, it is suggested that ligation of CD40 at the immunological synapse stimulates NF-κB-, but not JNK activation and thus favours DC survival (Kriehuber et al., 2005). This model complements our own findings that H_2O_2 induces JNK activation and thus apoptosis, but also that this outcome is enhanced by the absence of H_2O_2-induced NF-κB activation.

The mechanism of ROS-induced MAPK activation is not fully understood (Forman et al., 2004). Much of the previous work has been in neurons (Levinthal and Defranco, 2004). With respect to H_2O_2 specifically, there is much evidence for a role of Apoptosis Signal Regulating Kinase (ASK-1) in the induction of JNK activation (Tobiume et al., 2001; Huyer et al., 1997). The model proposes that ASK-1 is sequestered in an inactive state by the redox-sensitive thioredoxin protein. Upon exposure to oxidative stress, the conformation of thioredoxin is altered, since thioredoxin is cysteine rich and its oxidation results in the formation of many disulphide bridges. As a consequence, ASK-1 is released and activates JNK, which mediates the stress response and apoptosis (Saitoh et al., 1998). However, this model cannot be the major pathway for JNK activation in DC since inhibition of an alternative JNK activator, MLK, by CEP completely abrogates the activation of JNK by H_2O_2 (figure 3.9a). Furthermore, apoptosis is prevented by this inhibitor to almost the same degree as by the JNK-specific molecule SP, suggesting that upstream JNK activatory proteins, other than MLK, have minimal roles in H_2O_2-induced JNK activation in DC. MLK has been shown to be important in neuronal apoptosis induced by NGF-deprivation (Mota et al., 2001; Xu et al., 2001) supporting the hypothesis that MLK is the main regulator of H_2O_2-induced DC apoptosis.

H_2O_2 is known to be capable of oxidising cysteine residues, for example in the deactivation of thioredoxin, but the consequences of this depend on the molecular context, and therefore the pK_a, of the specific cysteine that is being oxidised. Most cysteines have an alkaline pK_a. In protein tyrosine phosphatases (PTPs), the catalytic cysteine interacts with other critical residues such that it's pK_a is reduced to acidic levels (pK_a 4.7-5.4), and therefore in these proteins, the catalytic cysteines exist as thiolate anions [cys-S^-] at neutral (or physiological) pH. This species is essential for the catalytic mechanism of phosphate removal in which a thiol-phosphate intermediate [cys-S-PO_3] is formed. Clearly therefore, the reversible oxidation of this thiolate by H_2O_2 (to sulphenic acid [cys-S-OH] (Salmeen et al., 2003; Forman et al., 2004)) will inhibit the action of PTPs transiently (Soberman, 2003).

Inhibition of PTPs allows tyrosine phosphorylation of intracellular proteins to proceed unchecked. The importance of this is underlined by the observation that active PTPs have a 100-1000 times higher turnover rate in comparison to tyrosine kinases (Reth, 2002), and thus signal propagation would continue unchecked unless PTPs terminate this signal. Inhibition of PTPs is therefore a very efficient mechanism for enhancing kinase signaling, and clearly this offers a potential mechanism for signal propagation by H_2O_2.

There is much evidence that inhibition of PTPs by H_2O_2 is a physiologically important event during cell signaling. Transient PTP inhibition by H_2O_2 has been suggested to play a

physiological function in regulating signaling by cell surface receptors in response to mitogens such as PDGF (Finkel, 2000), which initiates a signaling cascade involving tyrosine phosphorylation of the receptor. However, ligand binding also induces ROS generation, which amplifies signal propagation by transiently inhibiting the phosphatases that would otherwise terminate the signal prematurely (Meng et al., 2002). H_2O_2 has also been shown to inhibit PTP activity of CD45 in jurkat T lymphocytes (Secrist et al., 1993), and neutrophils (Fialkow et al., 1994), and PTP activity of SHP1 is inhibited by H_2O_2 (Cunnick et al., 1998).

This mechanism of MAPK activation in DC via PTP inhibition by H_2O_2 is supported by the extensive tyrosine phosphorylation observed following H_2O_2 treatment of DC (figure 3.12a), indicating unrestricted tyrosine phosphorylation. Furthermore, Na_3VO_4, an unrelated tyrosine phosphatase inhibitor (Huyer et al., 1997), also induces generalized tyrosine phosphorylation as well as specific phosphorylation of p38 and JNK (figure 3.12c). As with H_2O_2, a lower concentration of Na_3VO_4 is required to activate p38 than JNK.

We have examined the ability of three different stimuli to activate MAPKs in DC; LPS, H_2O_2, and Na_3VO_4, and all induce p38 activation at a lower concentration than JNK (figures 3.6 and 3.12). Such a pattern of p38 and JNK activation in DC has also been reported with other stimuli (Bunyard et al., 2003; Aiba et al., 2003; Nakagawa et al., 2004).

This greater sensitivity of p38 than JNK to external stimuli requires interpretation in a physiological context. As has been described, the role of the p38 pathway is to induce DC maturation, whereas we have identified a role for JNK in the control of DC apoptosis. Via its ability to activate MAPKs, the role of H_2O_2 is to enhance the level of activation of these pathways induced by conventional stimuli, perhaps by inhibiting the phosphatases that deactivate them. Any additive effect of H_2O_2 on p38 activation induced by other stimuli is not transduced into increased DC maturation due to the inability of H_2O_2 to activate NF-κB as discussed above. However, in the case of JNK, H_2O_2 is capable of synergising with other stimuli in the induction of JNK activation (figure 3.7), and there appears to be a threshold level of phosphorylated JNK above which death is induced (figure 3.8).

These observations can be extrapolated in to a more general model for the role of ROS and stress-induced MAPK activation in DC regulation. In order to optimally perform their sentinel role, DC reside at the epicentre of any infectious site, in contact with many different stimuli and stresses, including not only microbial stimuli but also more generalized tissue damage (Gallucci and Matzinger, 2001; Ibrahim et al., 1995). The DC integrate all of these signals, and

in the correct conditions, activate a maturation/ migration program. The activation of p38 is a key step in this process.

However, inflammation, the hallmark of infection carries its own inherent "dangers". In particular, ROS (and reactive nitrogen species), are likely to be present at significant concentrations, and are highly reactive molecules, which can cause alterations to nucleic acids, proteins and lipids. These altered moieties will themselves feed back on to the DC. A response by a DC that has been too badly damaged by ROS exposure may trigger an undesirable, or defective output. Modification of macromolecules by ROS will also lead to the formation of many "neo-epitopes" to which the host is not tolerant, and which, if presented effectively, will generate an entirely unwanted anti-self response, with possible pathological consequences.

Therefore in our model, ROS combine with other stimuli to activate JNK to reflect the level of "danger" to which the DC is being exposed. Above a threshold (figure 3.13, right-hand pathway) there is a "safety valve" to limit DC function, via the induction of apoptosis.

ROS will be present in the highest concentrations in the very centre of an inflammatory microenvironment, and if any DC are induced to undergo JNK-driven apoptosis due to possible damage, it will be those located at this site (figure 3.13, right-hand pathway). Due to the high reactivity of ROS, they are very short-lived and diffuse only very short distances. Therefore at sites adjacent to the area of highest ROS production, there will be less potential for damage to DC, or other cells, but significant levels of pathogen-derived material. We therefore propose that it is the DC originating from these locations, at much less risk from ROS-induced damage, and less likely to internalise the fragments of ROS-damaged self-cells, which will undergo p38-driven maturation (figure 3.13, left-hand pathway).

Unfortunately, due to the difficulty in determining the precise site of activation of a responding DC, it is not possible to obtain supporting evidence for this model from published data in mice or other systems. Indeed, supporting evidence for this model will require sophisticated methods to monitor the precise site at which DC are activated to migrate to draining lymphoid tissue.

Previous studies have supported a model in which the level of antigen determines the Th1/ Th2 bias of the resulting immune response (section 1.3.2.3). Accordingly, low concentrations of antigen induce prolonged DC survival and a Th2 response, whereas higher pathogenic doses induce p38-driven IL12 production and Th1 responses. We now propose an extension to this

model, in which, at still higher levels of stimulation, driven by high concentrations of inflammatory ROS production, DC do not mature but are induced to undergo JNK-induced apoptosis. This mechanism is postulated to limit the dangers of autoimmunity and/ or aberrant immune stimulation associated with over-activation of innate immunity.

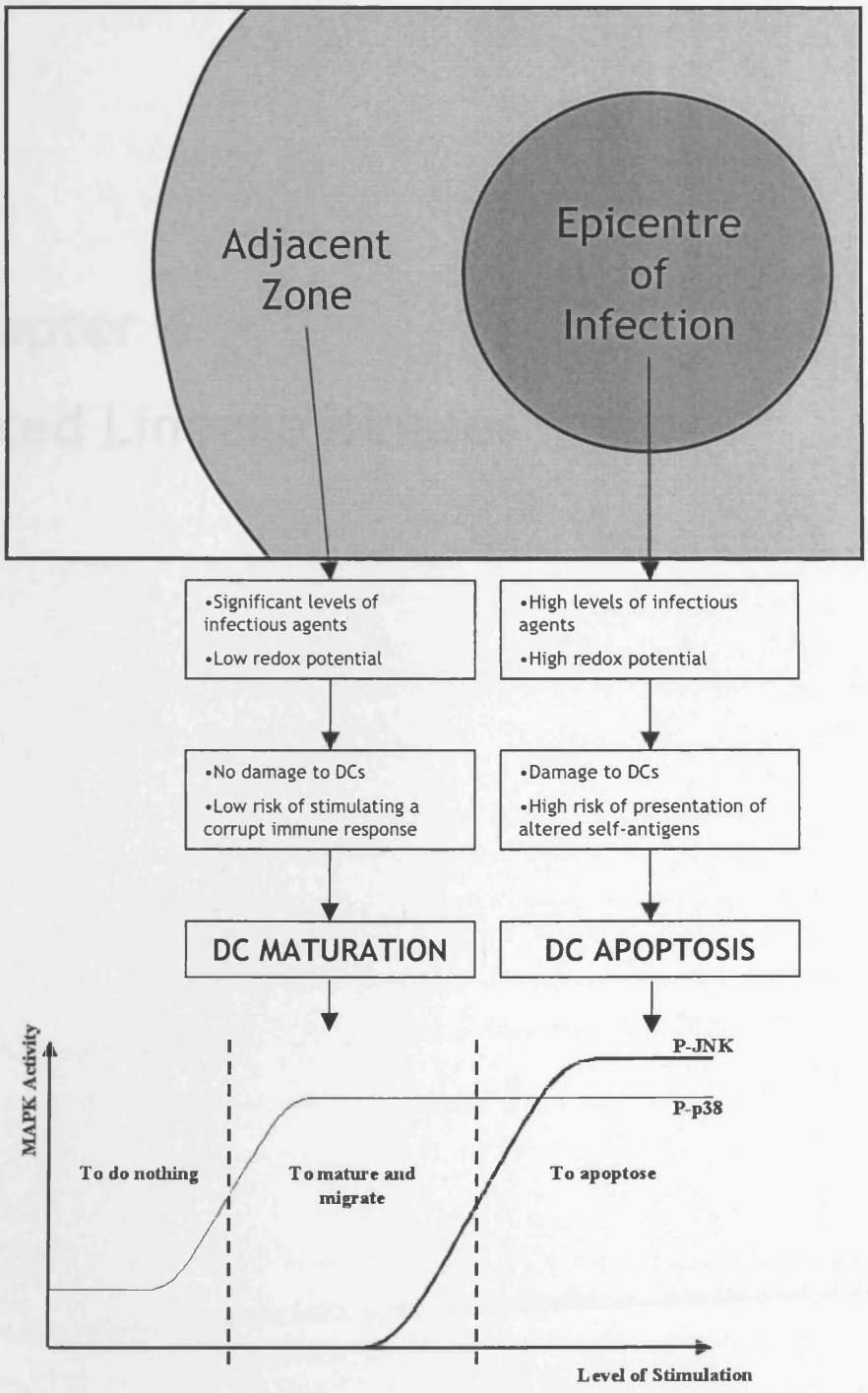


Figure 3.13. Model for the differential role of p38 and JNK in DC maturation versus apoptosis

Chapter 4

Mixed Lineage Kinases

4.1 Introduction

4.1.1 Mixed Lineage Kinases

In chapter 3 the role of JNK in DC maturation and apoptosis was probed using two small molecular weight inhibitors, SP, a direct inhibitor of JNK, and CEP, an inhibitor of an upstream kinase family, the mixed lineage kinases (MLKs). As discussed in section 1.3, MLKs are a family of serine/ threonine kinases, which act as MAPK kinase kinases (MAP3K) (Gallo and Johnson, 2002). Most previous work on MLK proteins (especially MLK3) has been in neuronal cells, limited to their selective activation of JNK, with an important consequent role in the regulation of apoptosis (Wang et al., 2004). Accordingly, an analogous role in regulating apoptosis in response to oxidative stress was demonstrated in DC in the previous chapter.

More recently, however, MLKs have been implicated in a variety of other cellular functions, including cytokine secretion (Falsig et al., 2004), cell division (Swenson et al., 2003), and morphology and migration (Hirai et al., 2002). Their involvement in signaling pathways is also more complex than was first thought, since MLK3 has also been implicated in the upstream regulation of p38 and ERK MAPKs, and even the NF-κB pathway (Hehner et al., 2000), in addition to JNK (Chadee and Kyriakis, 2004).

The signaling pathways downstream of TLRs are currently the focus of intense research, and many of the components of these pathways have been studied using knockout mice. The vast majority of these studies examine the activation of NF-κB as an end-point, and therefore the activation of p38 and JNK has only been examined in a relatively limited fashion. As a result, the mechanism of activation of these MAPKs by TLR ligands is not fully understood. Many MAP3Ks have been implicated in this process, including Tak1, ASK1, MEKK1, and MEKK3, (Irie et al., 2000; Into and Shibata, 2005; Matsuguchi et al., 2003; Huang et al., 2004), and we therefore decided to investigate whether MLK proteins were also involved in this process.

4.1.2 Chapter aims

In the previous chapter we observed that inhibition of MLK prevented H_2O_2-induced p38 activation in DC (figure 3.9). However, while H_2O_2-treatment did not induce DC maturation, the activation of p38 is known to be a critical step in the induction of DC maturation by other stimuli such as TLR ligands. We therefore wished to examine whether MLK functions in DC to activate p38 in response to TLR ligation.

Three stimuli were chosen, LPS, poly (I:C), and zymosan, due to their strong capacity to activate DC, and the known differences in the intracellular signaling mechanisms of their receptors. LPS is a constituent of the cell wall of many gram-negative bacteria, and is known to ligate TLR4 (Qureshi et al., 1999), in addition to several non-signaling accessory receptors such as CD14. The purity of the LPS used in this study was not tested, and it is therefore not impossible that additional signaling pathways were not activated in parallel to TLR4.

Poly (I:C) is a synthetic double stranded RNA mimic, which activates TLR3 (Alexopoulou et al., 2001). It is important to mention that poly (I:C) has also been suggested to induce type I interferon production via the cytosolic dsRNA-binding enzyme protein kinase R, independently of signalling through TLR3 (Diebold et al., 2005).

Zymosan is an insoluble preparation of yeast cell walls, primarily composed of beta-glucans, and activates DC via a heterodimer of TLR2 and TLR6 (Frasnelli et al., 2005). Due to the inexact constitution of zymosan preparations, it is likely that several different receptors will be stimulated, and in particular lectins which are strongly agonised by beta-glucans. Indeed, zymosan has also recently also been demonstrated to signal through the C-type lectin, Dectin1 (Rogers et al., 2005), although the relative contributions of the lectin and TLR receptor systems has not been comprehensively investigated.

Since the expression of MLK proteins in DC has not previously been examined, we also investigated the expression of different MLK isoforms in DC.

4.2 Results

4.2.1 Expression of MLK isoforms in DC

No data on the expression, activation, or function of MLKs in DC has previously been reported. We therefore wished to investigate which isoforms of the MLK family are expressed in DC, and to do this we used a nested PCR approach.

Our interest in the MLK family of MAP3Ks is largely due to the effects of the CEP inhibitor on DC function. As a priority, and a basis for further analysis, we therefore wished to investigate the expression of the inhibitor-sensitive MLK proteins rather than every member of the MLK family *per se*. As discussed in section 1.3.2, there are three families of MLK proteins, the MLKs, the DLKs, and the ZAKs. The CEP inhibitor is known to inhibit MLK1-3 of the MLK family. It also inhibits DLK, but not LZK, of the DLK family, however CEP does not inhibit the ZAK proteins, or other less closely related kinases such as PKC (Murakata et al., 2002). MLK4 has only recently been discovered. Indeed, it has not yet been shown to exist at the protein level *in vivo*. Because of this, the ability of CEP to inhibit MLK4 is not known. However, based on sequence similarity with MLK1-3, it is highly likely that CEP also inhibits MLK4, and for this reason we chose to also examine the expression of this enzyme in DC.

The design of the nested primer strategy was such that the PCR product crossed intron/ exon boundaries and thus distinguished the cDNA product from products arising from contaminating genomic DNA, due to different product sizes (figure 4.1). As a positive control for each PCR reaction we also amplified ß-actin.

Four PCR experiments were performed in parallel for each MLK protein with different templates:

- No template - to confirm the lack of contamination of any of the reagents.
- Genomic DNA - as a positive control to confirm that the primer pairs were all functioning correctly.
- Complementary DNA - to investigate the presence of the particular MLK transcript.
- Non-reverse transcribed RNA - to confirm the lack of contamination of the RNA.

A first round of PCR was performed using the outer primer pair on each of the four templates in parallel. In the second round, the nested primer pair was used both on the above four templates, and on the products of the first round reactions.

As shown in figure 4.2, PCR products of the correct size were obtained for MLKs 2, 3, 4, and DLK, but not MLK1 in immature DC. In each case, the identity of the product was confirmed by extraction from the gel and sequence analysis (data not shown).

4.2.2 Effects of TLR ligation on MLK3 phosphorylation

A fundamental question regarding MLK action in DC is whether these proteins are activated in response to DC maturation stimuli - this would clearly suggest a role in the regulation of the maturation process, and support the observations of the inhibitor studies.

As discussed in section 1.3, the activation of MLK proteins is both more complex and less completely understood than that of MAPKs. MLKs are believed to be phosphorylated at multiple sites, some of which are associated with enzyme activation while others are constitutive.

We next examined the expression and phosphorylation of MLK3, the most ubiquitous and extensively studied MLK (and hence the only MLK protein for which the appropriate reagents exist). The expression of MLK3 protein in DC was confirmed using polyclonal antibodies specific for total- and phosphorylated-MLK3. The antibody specific for phosphorylated-MLK3 was raised against a short polypeptide containing phosphorylated threonine and serine residues. This polypeptide corresponds to a section of the activatory loop of MLK3 at which phosphorylation at thr^{277} and ser^{281} in a T T X X S motif is thought to result in MLK3 activation (Leung and Lassam, 2001). The antibody was tested in a timecourse of DC treated with LPS (figure 4.3a), and indicated that MLK3 phosphorylation occurs in response to LPS, and was maximal after 45 minutes. This peak occurs slightly more rapidly than maximal phosphorylation of p38 or JNK, which is observed after 60 minutes - figure 4.4.

We then examined the ability of different concentrations of the three stimuli, LPS, poly (I:C), and zymosan to induce MLK3 phosphorylation. Phosphorylated MLK3 was observed even in unstimulated cells. The pattern of bands obtained were interesting, in that two bands were consistently observed, possibly representing phosphorylated and hyperphosphorylated forms of MLK3 (Swenson et al., 2003). The effect of stimulation upon the intensity of the two bands was variable. In response to LPS, the lower band is of a higher intensity than the upper band, both in response to a timecourse of stimulation (figure 4.3a) and at all LPS concentrations (figure 4.3b). In response to poly (I:C), however, the two bands are of equivalent intensity at all concentrations (figure 4.3c). In contrast, little, if any, MLK3 phosphorylation was observed with any concentration of zymosan (figure 4.3d).

4.2.3 Effect of MLK inhibition on activation of signaling pathways

Although originally described as a JNK-specific MAP3K (Rana et al., 1996), MLK3 has since been reported to also operate upstream of p38 (Gallo and Johnson, 2002; Tibbles et al., 1996), ERK (Chadee and Kyriakis, 2004), and NF-κB (Hehner et al., 2000). We therefore wished to investigate which of these downstream pathways are regulated by MLK in DC in response to different TLR ligands.

To determine the optimum incubation time to induce maximal signal pathway activity, a timecourse of LPS stimulation of DC was performed (figure 4.4). The results indicated that the p38, ERK, and JNK MAPK, and the NF-κB pathways were maximally activated 60 minutes after cell stimulation, therefore this incubation time was used in subsequent experiments. DC were then pre-incubated with 1μM of CEP, and then the ability of different concentrations of LPS, poly (I:C), and zymosan to activate the MAPKs and NF-κB was examined. Control experiments indicated that the CEP inhibitor alone had no effects on the activation of any of the pathways (data not shown), and did not affect viability of the cells at the concentration used (data not shown).

As shown in figure 4.5, MLK inhibition does not prevent activation of the ERK pathway in response to LPS, poly (I:C), or zymosan, in fact, slight enhancement of ERK phosphorylation was observed in the presence of the inhibitor. The degradation of IκBα is also not prevented by the inhibitor in response to any of the stimuli, suggesting that MLK does not regulate the canonical pathway of NF-κB activation in response to TLR ligation in DC.

Inhibiting MLK did not have the same outcome in terms of p38 and JNK inhibition in response to each stimulus. In response to both LPS and poly (I:C), CEP strongly inhibited the activation of p38 and JNK, however, in response to zymosan, very little effect on p38 activation was observed. JNK activation in response to LPS or poly (I:C) was also inhibited by CEP. Interestingly, however, JNK activation was not observed upon zymosan stimulation.

These data therefore suggest that the three stimuli activate MAPKs via distinct MAP3K pathways.

4.2.4 Effect of MLK inhibition on DC maturation

Given that MLK3 is activated by DC maturation stimuli (figure 4.3), that MLKs are upstream regulators of MAPKs in DC (figure 4.5), and that MAPKs have been shown to be important in

controlling DC maturation, we next investigated the effects of MLK inhibition on DC maturation.

DC were stimulated with LPS, poly (I:C), or zymosan and were then examined for surface protein expression in the presence of MLK and MAPK inhibitors. In addition to the CEP MLK inhibitor, inhibitors of the three MAPK pathways were used, SB and SP for p38 and JNK respectively, and PD98059 (PD), an inhibitor of ERK-specific MAP2Ks (Dudley et al., 1995).

Three primary phenotypic markers of DC maturation were chosen, reflecting the primary antigen specific signal (HLA-DR), a co-stimulatory signal (CD86) and a terminal differentiation/maturation signal (CD83). These cell surface markers have proved very consistent and reproducible cell surface indicators of DC maturation.

To determine the optimal concentration of LPS, poly (I:C), and zymosan to induce DC maturation, DC were incubated with different concentrations of the three stimuli, and the relative levels of maturation were determined by examination of CD86 and HLA-DR expression (figure 4.6).

The use of the CEP inhibitor has not been widely reported, therefore various concentrations of CEP were tested on LPS-induced CD86 upregulation, and 1μM was found to give maximal inhibition (figure 4.7).

The ability of the inhibitors alone to influence the expression of the three surface markers was examined, and none of the inhibitors were found to significantly alter the level of expression of any of the three proteins (figure 4.8).

The effect of the inhibitors on the expression of the three DC maturation markers, CD83, CD86, and HLA-DR, in response to stimulation with LPS, poly (I:C), or zymosan was then examined. All three markers were found to behave very similarly, both in terms of response to TLR ligation, and in terms of pharmacological intervention (figure 4.9). In response to LPS, poly (I:C) and zymosan there was clear evidence of maturation as judged by increase in CD83, CD86, and HLA-DR expression. 10μM of p38 inhibitor SB203580 was used as a result of findings in Chapter 3 in which this concentration was found to fully inhibit LPS-induced CD86 upregulation. Consistent with this, inhibition of p38 blocked upregulation of all three of the surface markers examined in response to all three stimuli. This observation confirms and extends previous studies emphasizing the central role for this MAPK in controlling DC maturation. In contrast, in extension to the observations of chapter 3, blocking the JNK

pathway did not significantly inhibit upregulation of any marker in response to any of the stimuli. ERK inhibition also had no effect on the stimulation-induced level of expression of the three markers.

In contrast to the MAPK inhibitors, the inhibitory activity of CEP was highly dependent on which TLR stimulus was tested. Upregulation of all three markers induced by LPS or poly (I:C) was blocked by the MLK inhibitor, to the same extent as by the inhibitor of p38 (figures 4.9a and b). In contrast, MLK inhibition had no effect on the upregulation of surface markers stimulated by zymosan (figure 4.9c).

4.2.5 Effect of MLK inhibition on cytokine secretion by DC

To further examine the effect of inhibiting MLK or MAPK on DC maturation, the secretion of three cytokines, TNF-α, IL10, and IL6 was studied.

Preliminary studies indicated a requirement for a larger number of DC per group than for the phenotype experiments in order for cytokine production to reach the detection limit of the assay. For this reason, we decided to examine in parallel the effects of MLK and p38 inhibition only. These protein families appear to be both linked (figure 4.5), and involved in the regulation of DC maturation (figure 4.9). The ERK pathway has been suggested to be involved in the regulation of DC cytokine secretion (section 1.2.2.3), however, ERK does not appear to be downstream of MLK, since MLK inhibition does not prevent ERK phosphorylation (figure 4.5). JNK phosphorylation is regulated by MLK (figure 4.5), but we had thus far observed no evidence for a role of JNK in the control of maturation - indeed zymosan did not induce JNK phosphorylation (figure 4.5c).

The SB and CEP inhibitors were found to have no significant effect on cytokine secretion when added to the DC in the absence of any other stimulus (figure 4.10).

As for cell surface phenotype, p38 inhibition by SB blocked secretion of all cytokines, in response to all stimuli, with one exception, i.e. the release of TNFα in response to zymosan (figures 4.11a, b, and c). In contrast, inhibition of MLK using CEP blocked cytokine secretion in response to LPS and poly (I:C), but had no inhibitory effect on any cytokine secreted in response to zymosan. In response to LPS, the degree of inhibition achieved by SB and CEP was similar, however, in response to poly (I:C), CEP had a greater inhibitory effect than SB on the secretion of all three cytokines.

To rule out that the selectivity between LPS and zymosan was simply a dose-related effect, the release of the three cytokines was tested at a series of different CEP inhibitor concentrations (figures 4.11d and e). A reduction in the secretion of all cytokines in response to LPS was seen even at the lowest concentration of CEP used. In contrast, no inhibition of the zymosan-induced response was seen at any concentration.

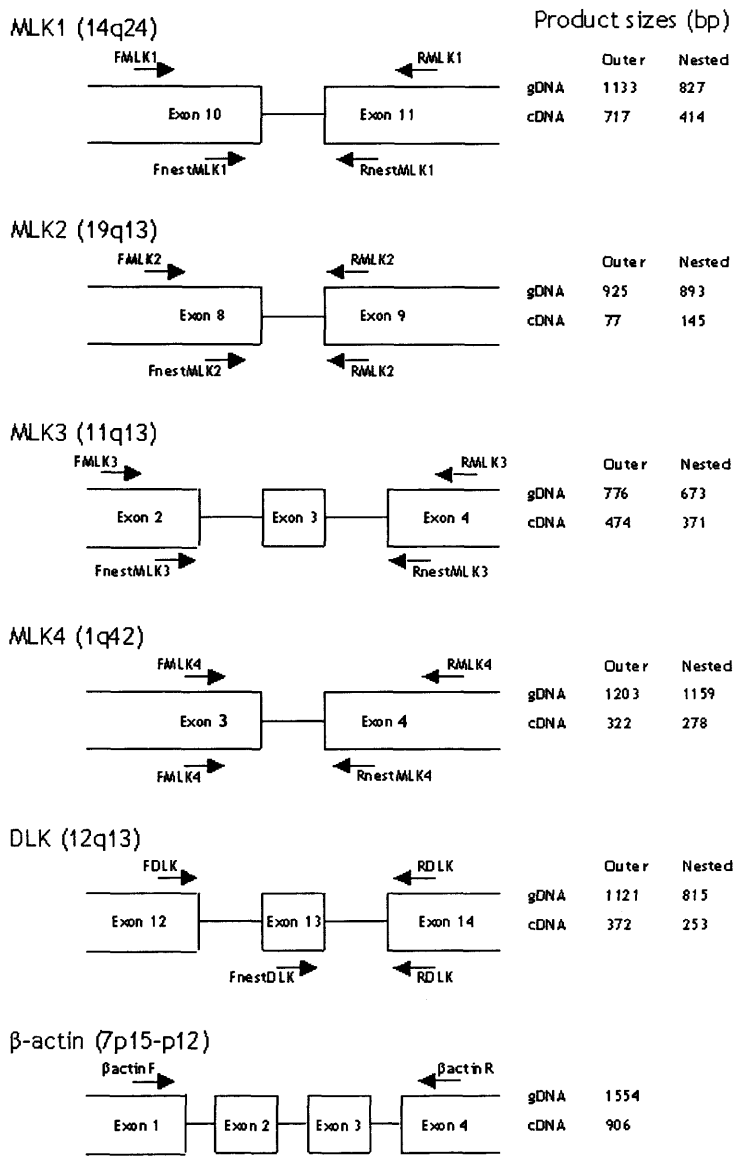


Figure 4.1. PCR Experimental design to assay MLK expression in human DC

Primers corresponding to known MLK isoforms were designed spanning small intronic regions of the genomic sequences to control for DNA contamination of the extracted RNA due to different sized products. To maximise stringency and sensitivity of detection of MLK protein synthesis, a nested strategy was used whereby one or two primers were designed to be used in a second PCR reaction using the first round product as template. B-actin primers were used as positive controls.

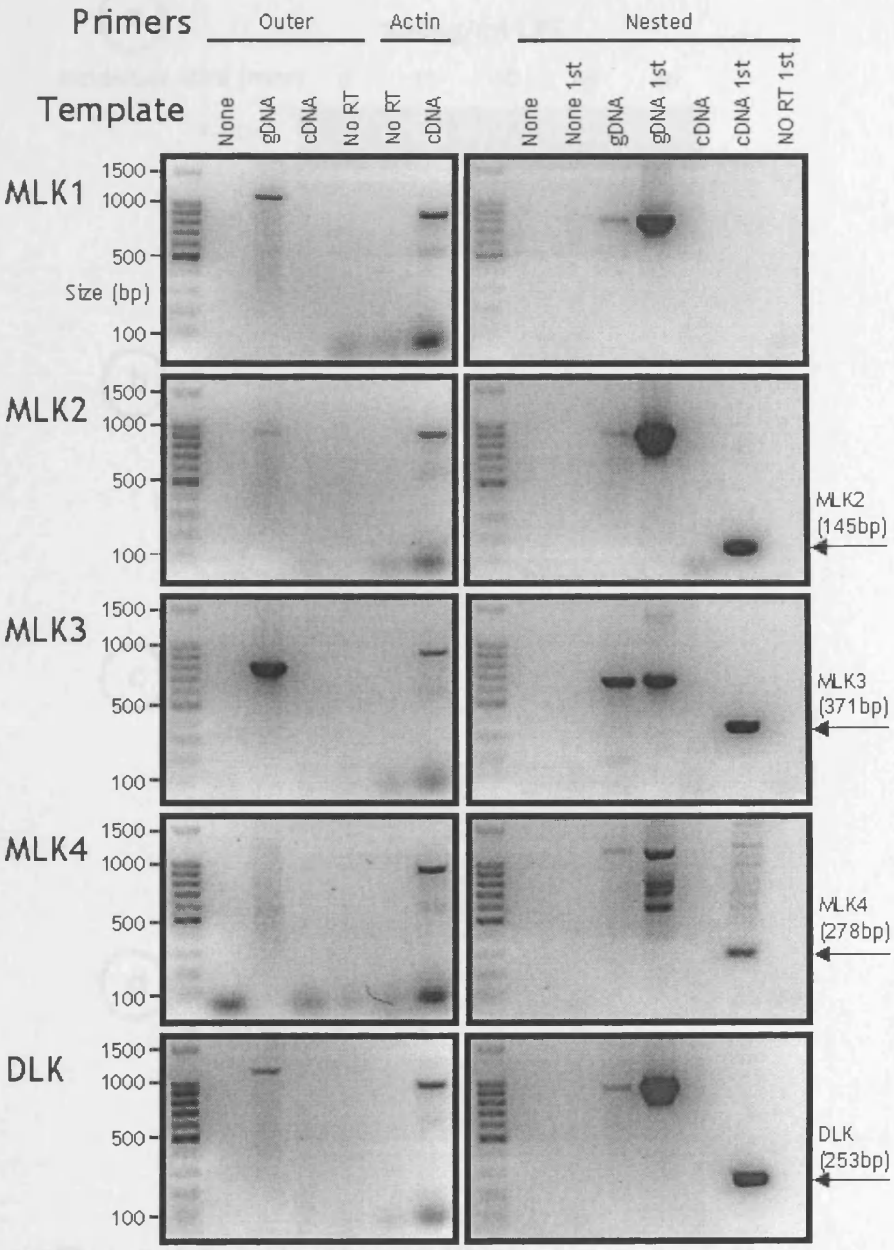


Figure 4.2. MLK isoform expression in human DC

Genomic DNA and total RNA were purified from immature DC and total RNA was then converted to cDNA. Left panels - first round of PCR (using the outer primers), right panels - second PCR cycle (using the nested primers). In both cycles the following templates were used: No template (to control for contamination of the primers or other components of the PCR reaction mixture), gDNA, cDNA, and non-reverse transcribed RNA. In the first PCR cycle, β-actin primers were used as control for RNA quality and purity. In the second cycle, products of the first cycle were used as templates in parallel to the particular template in question."1st" - product of first round PCR reaction used as template. Negative images shown.

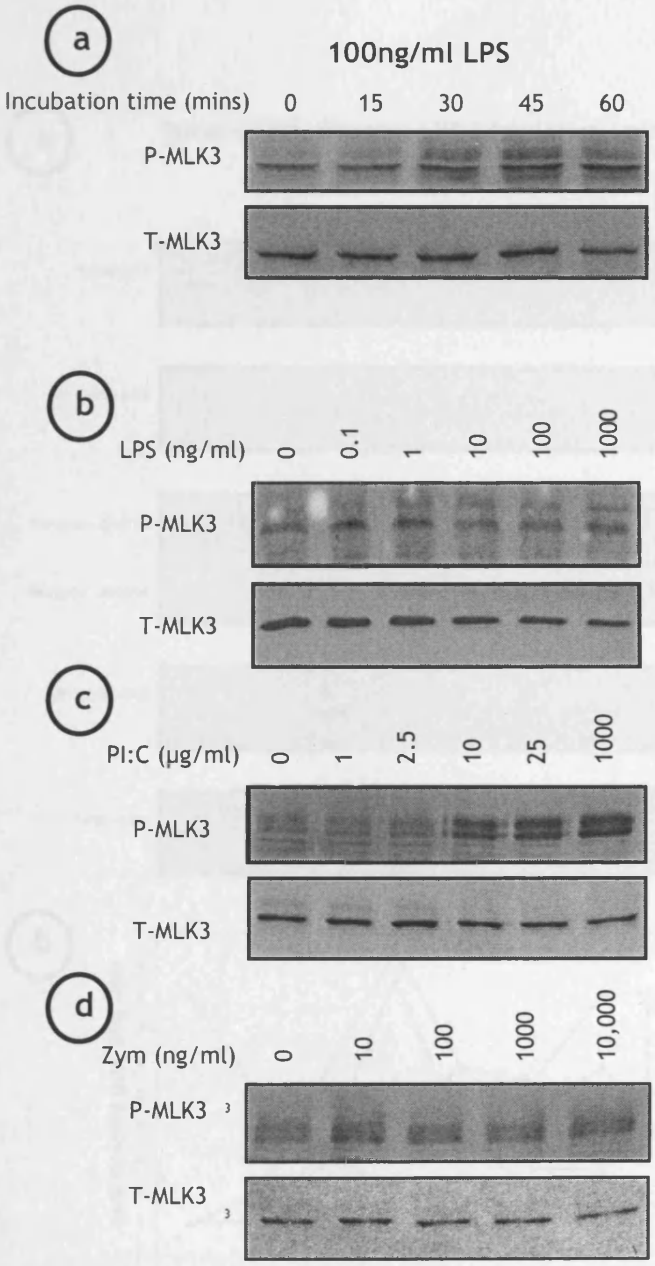


Figure 4.3. Induction of MLK3 phosphorylation by TLR ligands

(a) DC were treated with LPS (100ng/ml) and at various times post-stimulation cells were harvested, lysed and analysed by Western blot for the total and phosphorylated forms of the MLK3 as indicated. (b-d) DC were treated with various concentrations of (b) LPS, (c) Poly (I:C), or (d) Zymosan for 45 minutes as indicated and were then harvested, lysed and analysed by Western blot for the total and phosphorylated forms of MLK3 as indicated.

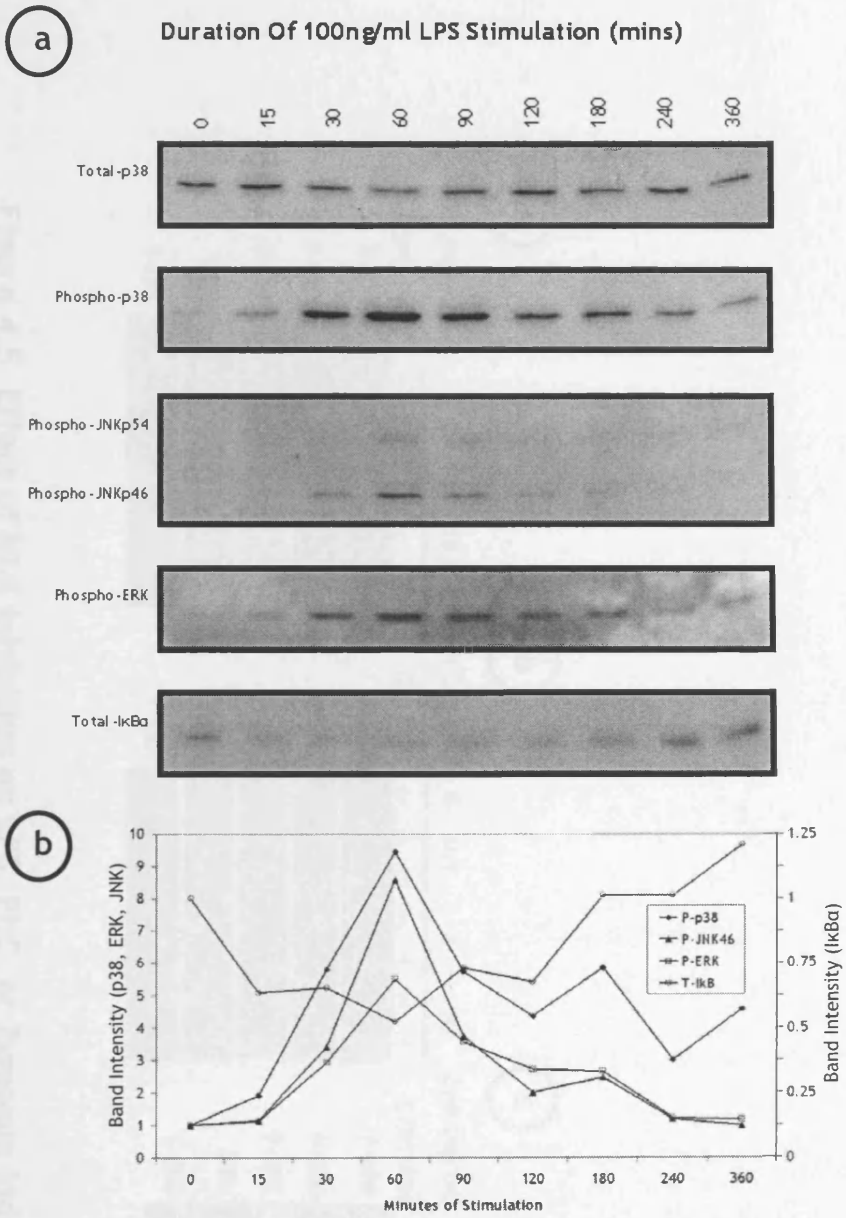


Figure 4.4. LPS-induced p38, JNK, ERK, and NF-κB activation in DC

(a) DC were treated with 100ng/ml LPS for various lengths of time as indicated. Cells were then harvested, lysed and analysed by Western blot for the Total or phosphorylated forms of p38, JNK, ERK, and IκBα as indicated. Representative of three independent experiments. (b) Densitometric analysis of (a). Band intensities were corrected to the intensity of the equivalent T-p38 band and then normalised to a value of 1 for the unstimulated (t=0) density (see materials and methods).

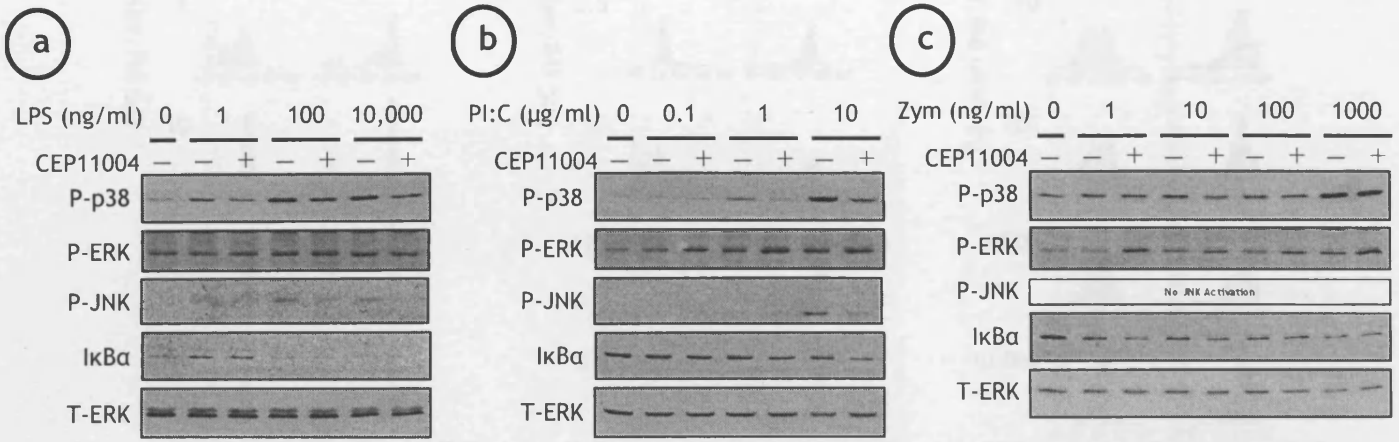


Figure 4.5. Effect of MLK inhibition on LPS, PI:C, or Zymosan induced MAPK activation

DC were treated for 18 hours with the MLK inhibitor CEP11004 (1μM) prior to stimulation with various concentrations of LPS (a), Poly (I:C) (b), or Zymosan (c) as indicated. At 60 minutes post-stimulation cells were harvested, lysed and analysed by Western blot for the phosphorylated forms of p38, ERK, and JNK MAPK, and total-IκBα as indicated. Representative of three independent experiments.

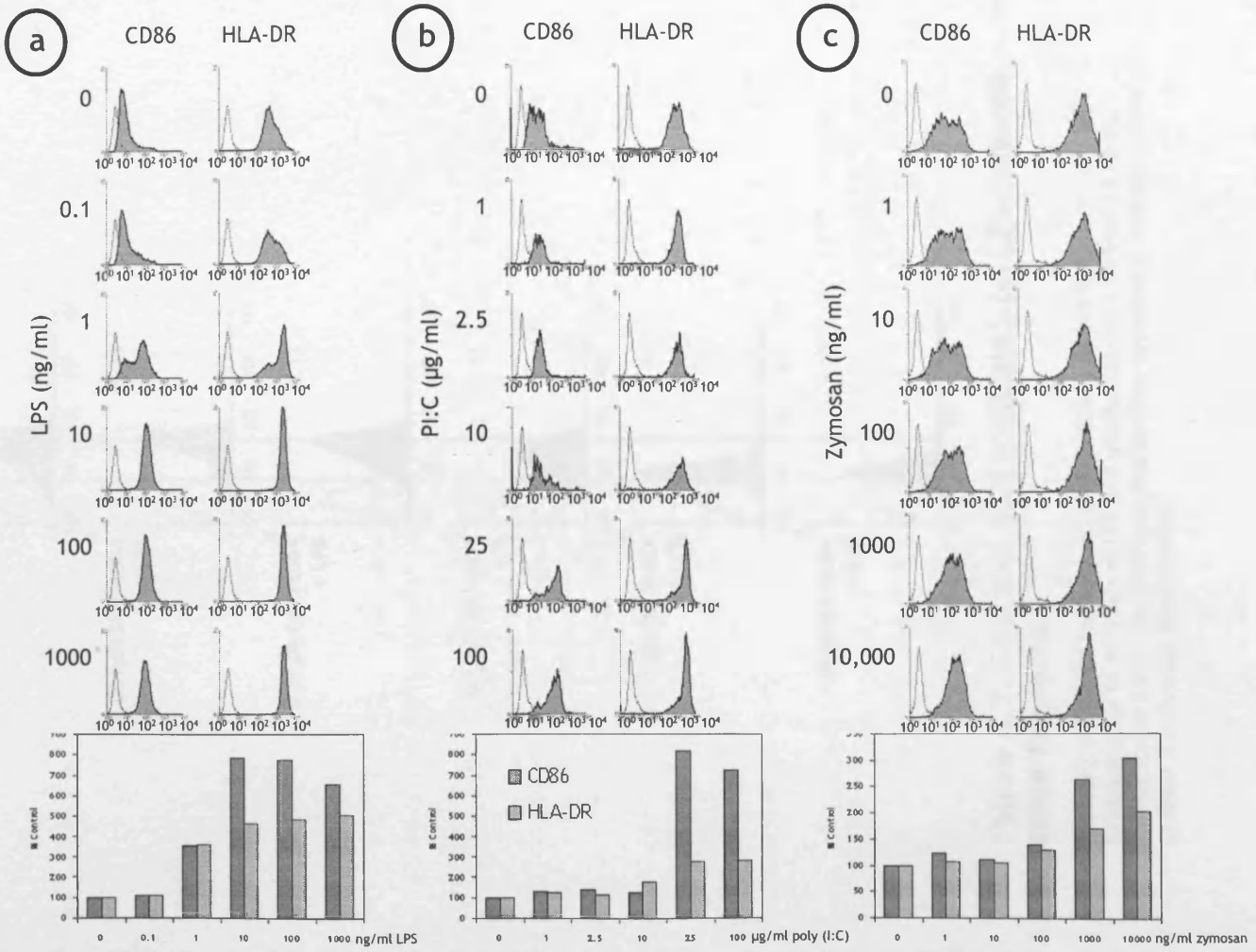


Figure 4.6. Effect of LPS, PI:C, and Zymosan on DC expression of CD86 and HLA-DR

DC were treated for 18 hours with various concentrations of LPS (a), Poly (I:C) (b), or Zymosan (c) as indicated. Expression of surface marker proteins was then analysed by flow cytometry. One representative (top panel), and normalised mean median fluorescent intensities (lower panels), of two independent experiments are shown.

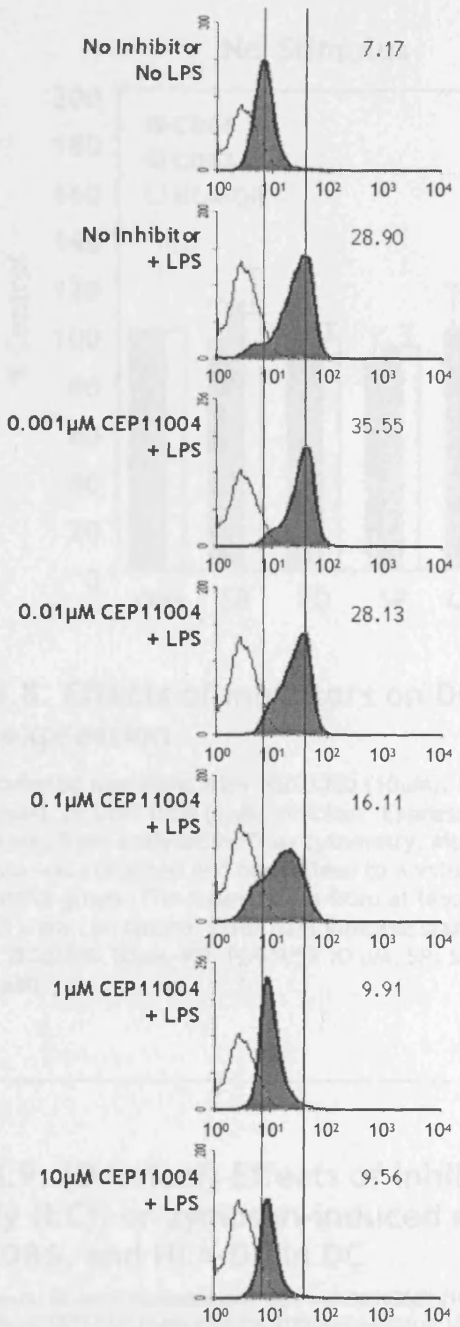


Figure 4.7. Effect of MLK inhibition on LPS-induced CD86 Expression

DC were incubated for 18 hours with various concentrations of CEP11004 inhibitor prior to addition of LPS and incubation for a further 18 hours. Expression of CD86 was then analysed by flow cytometry. Representative of at least 3 individual experiments.

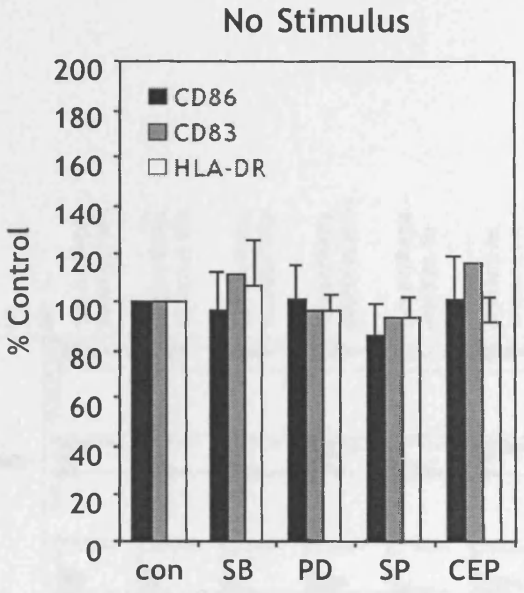


Figure 4.8. Effects of inhibitors on DC surface marker expression

DC were incubated overnight with SB203580 (10μM), PD98059 (10μM), SP600125 (1μM), or CEP11004 (1μM) inhibitor. Expression of CD83, CD86, and HLA-DR was then analysed by flow cytometry. Median fluorescence intensity data was collected and normalised to a value of 100% for the no inhibitor control group. The mean values from at least three individual experiments were calculated. Error bars indicate standard error of the mean. (SB; SB203580 10μM, PD; PD98059 10 μM, SP; SP600125 1μM, CEP; CEP11004 1μM).

Figure 4.9. (Overleaf) Effects of inhibitors on the LPS, poly (I:C), or zymosan-induced expression of CD83, CD86, and HLA-DR in DC

(a-c) Upper panels; DC were incubated overnight with SB203580 (10μM), PD98059 (10μM), SP600125 (1μM), or CEP11004 (1μM) inhibitor prior to addition of LPS (a), poly (I:C) (b), or zymosan (c) as indicated and incubated for a further 18 hours. Expression of CD83, CD86, and HLA-DR was then analysed by flow cytometry. Representative of at least 3 individual experiments. Lower panels; Analysis of the effect of MAPK and MLK inhibition on DC expression of CD83, CD86, and HLA-DR. Experiments were performed as described for the upper panels and median fluorescence intensity data was collected and normalised to a value of 100% for the stimulus control group. The mean values from at least three individual experiments were calculated. Error bars indicate standard error of the mean, and asterisks indicate $p<0.05$ versus stimulus control (calculated from pre-normalised MFI values). (SB; SB203580 10μM, PD; PD98059 10 μM SP; SP600125 1μM, CEP; CEP11004 1μM)

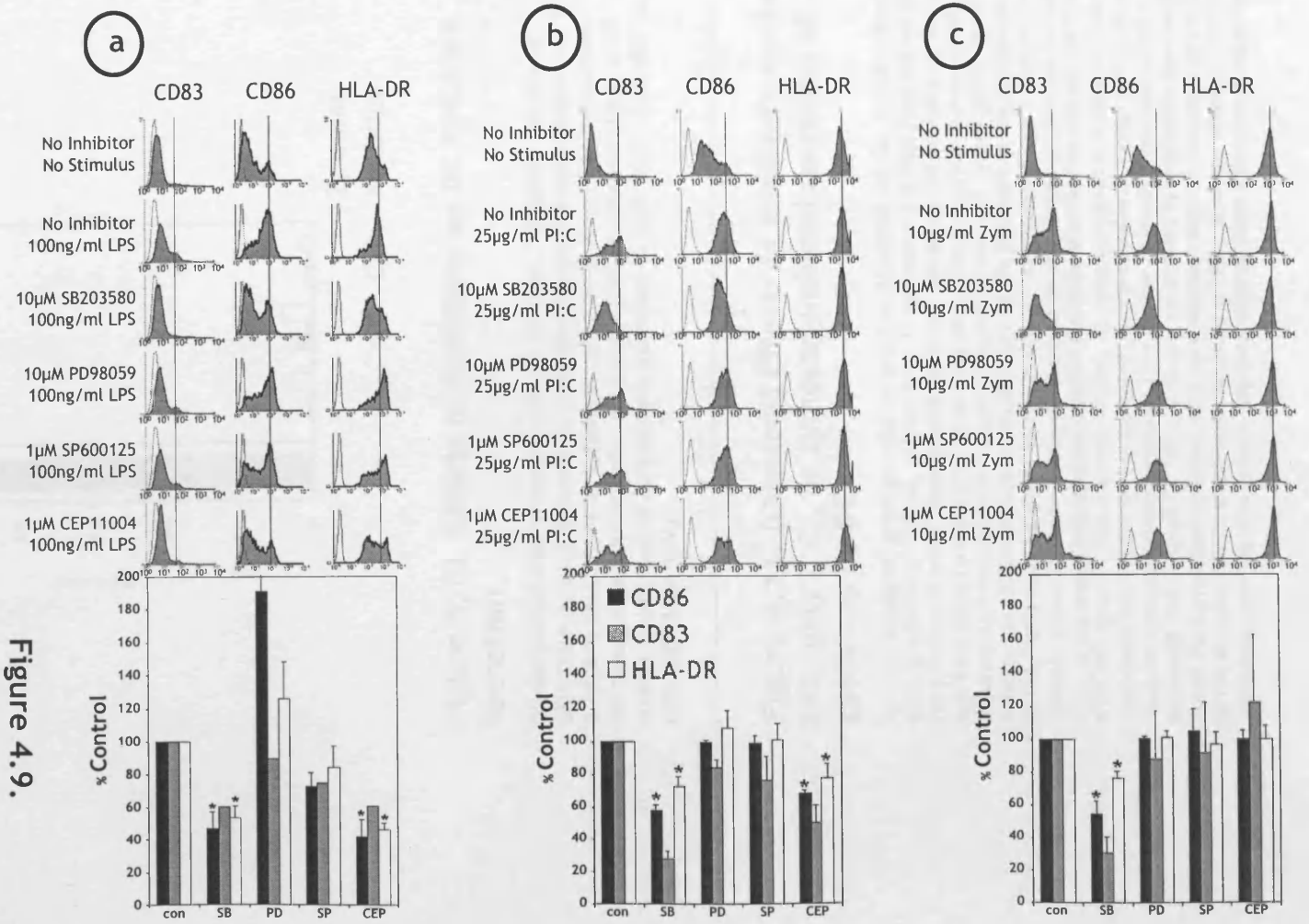

a
CD83
CD86
HLA-DR
No Inhibitor
No Stimulus
No Inhibitor
100ng/ml LPS
10μM SB203580
100ng/ml LPS
10μM PD98059
100ng/ml LPS
1μM SP600125
100ng/ml LPS
1μM CEP11004
100ng/ml LPS
% Control
con
SB
PD
SP
CEP
b
CD83
CD86
HLA-DR
No Inhibitor
No Stimulus
No Inhibitor
25μg/ml PI:C
10μM SB203580
25μg/ml PI:C
10μM PD98059
25μg/ml PI:C
1μM SP600125
25μg/ml PI:C
1μM CEP11004
25μg/ml PI:C
CD86
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Figure 4.9.

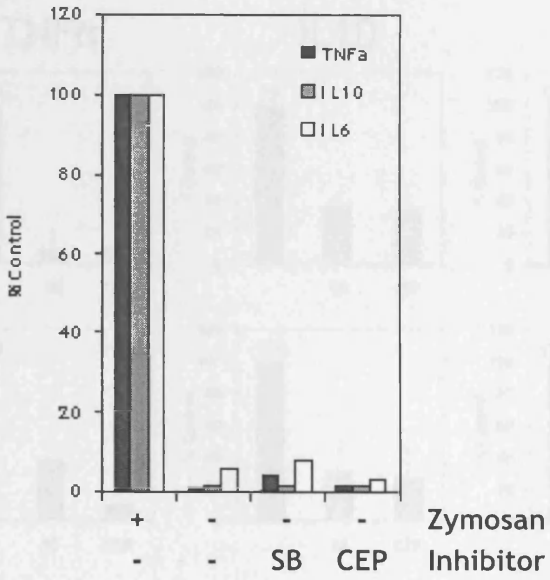

Figure 4.10. Effects of inhibitors on DC cytokine secretion

DC were incubated overnight with no inhibitor, SB203580 (10μM), or CEP11004 (1μM). Supernatants were then harvested and analysed for TNFα, IL10, and IL6 content by cytometric bead array assay. For comparison purposes, data were adjusted such that cytokine concentrations of DC treated with 10μg/ml Zymosan are indicated as 100%. (SB; SB203580 10μM, CEP; CEP11004 1μM).

Figure 4.11. (Overleaf) Effects of inhibitors on the LPS, poly (I:C), or zymosan-induced secretion of cytokines by DC

DC were incubated overnight with no inhibitor, SB203580 (10μM), or CEP11004 (1μM) prior to addition of LPS (a), poly (I:C) (b), or zymosan (c) as indicated and incubated for a further 18 hours. Supernatants were then harvested and analysed for TNFα, IL10, and IL6 content by cytometric bead array assay. For comparison purposes, data were adjusted such that cytokine concentrations of the stimulus control group were indicated as 100%. Absolute values of cytokines are indicated above the control bars in pg/ml. Note; absolute values for LPS groups are not comparable with poly (I:C) or zymosan groups. Mean of 3 independent experiments. Error bars indicate standard error of the mean, and asterisks indicate $p<0.05$ versus stimulus control (calculated from raw cytokine concentration values). DC were incubated overnight with various concentrations of CEP11004 as indicated prior to addition of 100ng/ml LPS (d) or 10μg/ml Zymosan (e) and incubation for a further 18 hours. Supernatants were harvested and analysed for TNFα, IL10, and IL6 content by cytometric bead array assay. For comparison purposes, data were adjusted such that cytokine concentrations of the stimulus control group were indicated as 100%. Absolute values of cytokines are indicated above the control bars. Representative of three independent experiments.

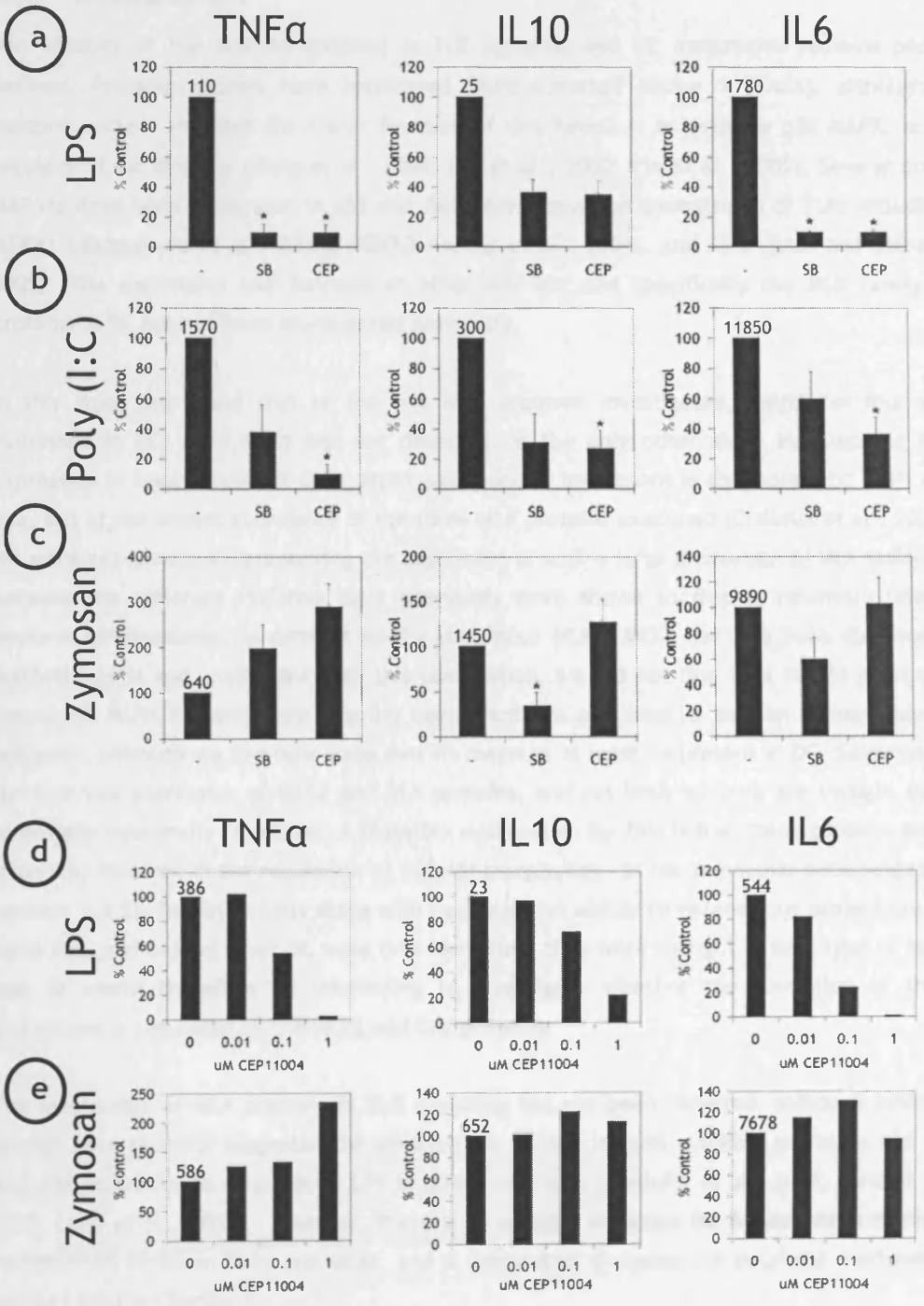
a
TNFα
IL10
IL6
LPS
% Control
110
25
1780
b
Poly (I:C)
1570
300
11850
c
Zymosan
640
1450
9890
SB
CEP
d
TNFα
IL10
IL6
LPS
386
23
544
e
Zymosan
586
652
7678
0
0.01
0.1
1
uM CEP11004

Figure 4.11.

4.3 Discussion

The identity of the MAP3Ks involved in TLR signaling and DC maturation remains poorly defined. Previous studies have implicated TGFβ-activated kinase 1 (TAK1), although it remains unclear whether the major function of this kinase is to activate p38 MAPK, or to regulate NF-κB directly (Jiang et al., 2004; Irie et al., 2000; Irie et al., 2000). Several other MAP3Ks have been implicated in p38 and JNK MAPK activation downstream of TLRs including, MEKK1 (Matsuguchi et al., 2003), MEKK3 (Huang et al., 2004), and ASK1 (Into and Shibata, 2005). The expression and function of other MAP3Ks, and specifically the MLK family of proteins in DC has not been investigated previously.

In this study we found that of the five MLK enzymes investigated, mRNA for four was expressed in DC. Only MLK1 was not detected. In the only other study investigating MLK expression in haematopoietic cells, MLK1 was found to be present in the monocytic THP1 cell line, but at the lowest abundance of the three MLK proteins examined (Ciallella et al., 2005). We were not anticipating observing the expression of such a large proportion of MLK isoforms, because the different isoforms have previously been shown to display relatively limited patterns of expression. In contrast to the ubiquitous MLK3, MLK1 has only been observed in epithelial cells and, consistent with this observation, we did not find it in DC. As previously described, MLK4 has only very recently been identified and thus its pattern of expression is unknown, although we can now state that its message at least, is present in DC. Surprisingly, we observed expression of MLK2 and DLK proteins, and yet both isoforms are thought to be essentially neuronally restricted. A plausible explanation for this is that these proteins are in some way involved in the regulation of cellular morphology, as has previously been suggested (section 1.3.5). Dendritic cells share with neurones the ability to extend long projections, or dendrites, and indeed when DC were first identified, they were thought to be a type of nerve cell. It would therefore be interesting to investigate whether the formation of these projections is regulated by the MLK2 and DLK proteins.

The mechanism of MLK proteins in TLR signalling has not been reported, although inhibitor studies have recently suggested the involvement of MLK in both cytokine secretion and p38 and JNK activation in response to LPS in other cell types (Ciallella et al., 2005; Lund et al., 2005; Lund et al., 2005). However, there is no existing evidence for a mechanism of signal transmission between TLRs and MLKs, and a speculative discussion of potential mechanisms can be found in Chapter 6.

In this study a link between at least one member of the MLK family, MLK3, and TLR signaling was demonstrated by showing that MLK3 is phosphorylated in response to the TLR4 ligand LPS,

and the TLR3 ligand poly (I:C). In contrast, the TLR2/6 ligand zymosan showed only a very slight transient effect on MLK3 phosphorylation. The precise role of phosphorylation in controlling MLK3 activity is complex and not well understood, and may reflect a combination of auto-phosphorylation and para-phosphorylation by kinases including AKT2 (Figueroa et al., 2003) and germinal centre kinases (Chadee et al., 2002). The phosphorylation induced in response to TLR ligation, was not blocked by the MLK inhibitor CEP (not shown) suggesting that an additional kinase was involved linking the ligation of TLR to MLK activation, rather than simply an autophosphorylation event. An alternative explanation is that the CEP inhibitor functions in such a way that autophosphorylation of MLK proteins is not prevented, whereas the subsequent phosphorylation of substrate proteins is inhibited. We consistently observed two bands when immunoblotting for phosphorylated MLK3. It is possible that one of these bands represents a non-MLK3-specific target of the antiserum. However, 12 potential phosphorylation sites have been observed in MLK3 (Vacratsis et al., 2002), and so it is possible that phosphorylation at the activatory loop induces further phosphorylation events, resulting in a hyperphosphorylated MLK3 species possessing a slightly different electrophoretic mobility. Indeed, hyperphosphorylation of MLK3 has previously been demonstrated (Swenson et al., 2003).

The timecourse of activation, which peaked after approximately 45 minutes (figure 4.3), was consistent with a function in downstream activation of MAPKs, since maximal phosphorylation of p38 and JNK is observed slightly later, at around 60 minutes post-stimulation (figure 4.4). The temporal link between MLK and MAPK activity was further supported by pharmacological studies using the selective MLK inhibitor CEP. In agreement with several other studies, inhibition of MLKs interfered with downstream signaling via both the p38 and JNK MAPK pathways (figure 4.5). Remarkably, the involvement of MLK was again very stimulus dependent. Inhibition of MLK blocked p38 and JNK activation in response to both LPS and poly (I:C), but had little or no effect on p38 activation by zymosan. Thus activation of p38 is a common end-point in signaling via TLR3 and 4, and TLR2/6, but is mediated via different up-stream pathways.

MLK inhibition did not prevent ERK activation in response to any of the stimuli, indicating that MLK does not regulate the activation of ERK in DC. Previous reports have also implicated MLK in the induction of IκBα degradation and consequent activation of NF-κB (Hehner et al., 2000), and we therefore investigated the ability of CEP to prevent IκBα degradation. CEP did not prevent degradation of IκBα, suggesting that in DC, MLK proteins do not regulate NF-κB activity.

The fact that JNK phosphorylation was not observed in response to zymosan (figure 4.5) lends support to the theory outlined in chapter 3 that the role of the JNK pathway in the control of DC maturation is minimal. Furthermore, whilst LPS and poly (I:C) induced a significant degree of apoptosis at the highest concentrations tested, no apoptosis was observed in response to zymosan at any concentration (data not shown), consistent with the hypothesis that JNK is involved in controlling stress-induced DC apoptosis (chapter 3). However, in the previous chapter, JNK activation was observed at higher concentrations than p38 in response to both LPS and H_2O_2 treatment of DC, so it may be that higher concentrations of zymosan than those used here are required to activate JNK. JNK, and in particular the JNK-sensitive transcription factor AP1 has been implicated in zymosan-dependent transcription in other cell types (Tran-Thi et al., 1995).

The bifurcation between TLR ligation and downstream signaling is further reinforced by examining the selective effects of the MLK inhibitor on DC function. Consistent with previous reports, no reduction of expression of CD83, CD86, or HLA-DR was observed in the presence of the ERK inhibitor PD, and indeed LPS-induced surface marker expression was enhanced, as has been reported previously (Puig-Kroger et al., 2001). In extension to the observations of chapter 3, JNK inhibition did not significantly inhibit upregulation of any of the surface markers in response to any of the stimuli, supporting the idea that JNK plays only a minimal role in controlling DC maturation. Inhibition of p38, however, reduces the upregulation of surface markers and the secretion of all cytokines in response to all three stimuli. The only exception was the induction of TNFα by zymosan on which p38 inhibition had no effect. This was a surprising observation given the apparently ubiquitous role of p38 in the control of DC maturation in response to all the stimuli that we had investigated. Further quantitative differences can be observed between the pharmacological profiles of all three stimuli (LPS, poly (I:C), and zymosan) suggesting the picture is even more complex, but these will require more detailed future analysis. Indeed, the regulation of cytokine production is multifaceted, and known to occur at several levels, including transcriptionally and post-transcriptionally, and therefore probably also includes cell-type-specific regulatory mechanisms. The CEP inhibitor prevented both the upregulation of surface markers and the secretion of cytokines induced by LPS and poly (I:C), and this is consistent with a previous report in which this inhibitor was shown to inhibit LPS-induced TNFα and IL6 secretion from THP1 monocytes, and *in vivo* (Ciallella et al., 2005). In contrast, pre-treatment of cells with the MLK inhibitor did not affect the ability of zymosan to induce upregulation of surface markers or cytokine secretion. Thus, whereas inhibition of p38 MAPK inhibits both cell surface phenotype changes and cytokine secretion in response to LPS, poly (I:C) and zymosan, inhibition of MLK blocks the responses to LPS and poly (I:C), but not Zymosan.

The difference in MLK dependence of different TLR pathways remains to be explained at a molecular level. It is important to emphasise that although the three stimuli used in this study are known to be strong TLR agonists, they may not be exclusively targeting TLR-induced signaling pathways (as outlined above). Indeed, this is one possible explanation for the dichotomy in outcomes obtained with the different stimuli. Assuming the ligands used are specifically stimulating TLRS, one attractive hypothesis is that the differences in MLK utilisation by LPS and poly (I:C) versus zymosan may reflect the utilization of different intracellular adaptor proteins by the TLRs. Analysis of mice deficient for different adaptor proteins indicates that TLR3 and TLR4, but not TLR2, posses a strong signaling requirement for the TIR-domain-containing adaptor inducing IFN-β (TRIF) protein (Yamamoto et al., 2003) (section 1.2.2.4). The observed effects are similar to those reported here and suggest that TRIF-dependent MAPK activation may operate via MLK. This hypothesis will be discussed in more detail in chapter 6.

Another explanation for the different MLK dependency of the three stimuli is that zymosan is known to ligate a second receptor on the surface of DC in addition to TLR2/6, called Dectin-1 (Rogers et al., 2005). Dectin-1 is a C-type lectin, which recognises carbohydrate moieties of zymosan and has been shown to be essential for IL2 and IL10 production via syk tyrosine kinase-induced intracellular signaling. The full implications of this signaling pathway in DC are not known, however it is clearly possible that by using this parallel pathway zymosan is able to by-pass MLK in activating p38.

The involvement of the MLK family in the regulation of DC maturation has not been previously reported. Our results are consistent with a role for MLKs as MAP3Ks, inducing the phosphorylation and activation of the downstream kinases p38 and JNK. However, the majority of the data presented in this chapter are based upon inhibitor studies, and thus require confirmation using alternative approaches. For example, RNAi technology may be used to delete specific MLK isoforms and thus further dissect the role of MLK proteins in the control of DC maturation. Such studies have previously been used to identify roles for MLK3 in ERK activation (Chadee and Kyriakis, 2004) and cell cycle regulation (Swenson et al., 2003). However, the homology between the members and the functional degeneracy that this homology may permit will make this approach very difficult. Indeed, an MLK3-deficient mouse has recently been reported, and demonstrated far fewer phenotypic aberrations than one might expect given the number and variety of putative MLK3 functions (Brancho et al., 2005). This clearly reflects the ability of the MLK proteins to functionally compensate for one another.

Despite the fact that the use of inhibitors is not without limitations (section 1.2.1), phosphorylation of MLK3 was observed in response to DC stimulation using phospho-specific antibodies. We feel that this observation underpins the data obtained with the CEP inhibitor. In particular, we found a much lower degree of MLK3 phosphorylation in response to zymosan relative to LPS or poly (I:C), and this complements the inhibitor data, which reflects a significant role for MLK in the induction of DC maturation by LPS and poly (I:C), but not zymosan.

The most important finding, however, is the demonstration that different TLRs utilize different MAP3K to activate their respective intracellular pathways. The identity of the TLR agonist is one of several factors important in determining the T cell polarization of the resulting immune response and clearly the differential involvement of signal transduction proteins such as the MLKs may regulate the polarity of this outcome. TLR2 ligands have previously been implicated in the induction of Th2 responses (Agrawal et al., 2003; Re and Strominger, 2001), although many other environmental and stimulus-specific factors are known to be important. It is noteworthy that LPS and poly (I:C) induced the secretion of a larger amount of the proinflammatory cytokine TNFα than the regulatory factor IL10, whereas in contrast zymosan induced the production of more IL10 than TNFα (figure 4.11). This suggests that LPS and poly (I:C) are responsible for the induction of proinflammatory Th1-type responses, whereas zymosan has a more regulatory, or possibly Th2-inducing effect, as has previously been described (Qi et al., 2003). To confirm this, it would be interesting to examine the ability of the three stimuli to induce IL12 production, and also examine the cytokine secretion profiles of responding T cells. However, with the regards the data presented here, it is tempting to speculate that the differential role for MLK proteins that we have observed in the responses to LPS and poly (I:C) versus zymosan in some way mediates this effector T cell bias. Further studies, in particular using a larger selection of stimuli, including Th2-inducing ligands such as SEA or Pam-3-cys, would be required to investigate this hypothesis further.

In support of the observations presented here regarding the utilisation of different signaling pathways by zymosan and LPS, these stimuli have previously been shown to induce TNFα and IL6 by different mechanisms. While LPS-induced TNFα and IL6 production in macrophages is inhibited by overexpression of IκBα and thus NF-κB inhibition, the zymosan-induced production of these cytokines is not, although both stimuli induce significant amounts of both cytokines (Bondeson et al., 1999).

Both functional studies and transcriptional profiling (Kaisho and Akira, 2004; Moschella et al., 2001; Messmer et al., 2003) have clearly documented that the response to different pathogens in DC involves a common "core" response, but also a degree of plasticity capable of tailoring the response to the pathogen. Other studies have demonstrated that different TLRs associate preferentially with different adaptors. The present study suggests a possible role for MLKs as another intracellular step defining the specificity of different TLR signals.

Chapter 5

Morphology

5.1 Introduction

5.1.1 Introduction

In chapter 4 we found that inhibiting MLK proteins using the CEP inhibitors prevented LPS-induced DC maturation in terms of both upregulation of surface markers and secretion of cytokines. As described in section 1.1.3, however, DC maturation *in vivo* is also accompanied by changes in DC morphology, with initial retraction of processes, followed by migration to lymphoid tissue, and then formation of the typical dendrites, seen in interdigitating DCs (Steinman et al., 1997). The ability of DC to endocytose and phagocytose is reduced during this maturation phase. *In vitro* this morphological maturation is reflected by an increased capacity to extend dendritic projections.

The cells used in this thesis as a DC model are differentiated from peripheral blood mononuclear cells (PBMCs) (Sallusto and Lanzavecchia, 1994), and as demonstrated in chapter 3, these cells closely resemble immature DC. In the quest to model the *in vivo* scenario as closely as possible, an experimental protocol was recently described whereby these DC were cultured on the extracellular matrix (ECM) protein fibronectin (FN) coated on glass coverslips. Under these conditions these cells show a greater capacity to adhere than to glass or to tissue culture plastic alone. Furthermore, a greater proportion of cells display long dendritic processes, closely resembling the *in vivo* morphology (Swetman et al., 2002).

5.1.2 Chapter aims

In chapter 4, the effects of MLK and MAPK inhibitors on DC maturation in terms of altered phenotype and cytokine secretion were described. In this chapter, we aimed to investigate the effects of these inhibitors on the maturation of DC adhered to FN substratum with respect to morphology and endocytic capacity.

To facilitate these investigations, a subsidiary aim arose, because it was necessary to develop a novel method of analysing the capacity of DC to bind and internalise antigen in different morphological phases.

Correlative quantitative analysis of binding and internalisation by monocyte-derived myeloid DC as used in these studies is sparse. DC adhered to FN substratum and exhibiting a typical *in vivo* morphology provide a very useful starting point for such studies. Most current approaches to assaying endocytic/ phagocytic capacity of DC are based upon on flow cytometric analyses of cells in suspension that have internalised fluorescent material such as opsonised latex

beads or FITC-conjugated dextran (Lehmann et al., 2000; Dunn and Tyrer, 1981). However, these assays do not permit correlation of uptake with morphology.

Although the advent of confocal microscopy has made this an easier task than previously, nevertheless, current in situ fluorescent microscopy techniques are not quantified easily. Due to the small size of the particles used, most are limited to distinction between cells that are positive and negative for uptake (Hampton and Winterbourn, 1999). Furthermore, it is often difficult to discriminate between internalised and externally bound particles (Hampton and Winterbourn, 1999). An additional drawback of most current assays of phagocytosis is that degradation of probes in the endocytic pathway can result in artificially low measurements of phagocytic capacity, as measured either by flow cytometry or fluorescent microscopy (Lehmann et al., 2000). To overcome this problem, fluorescent dyes may be rendered insensitive to pH by incorporation into polystyrene beads; however, this involves a complex spray-drying technique (Thomasin et al., 1998).

In brief outline (see section 2.9 for details), coating of microbeads with PE-conjugated anti-DC-SIGN antibody enhances targeting to the surface of cells. Following an incubation period, secondary staining with a FITC-conjugated antibody allows distinction between internal and external beads. The microbeads used have a larger diameter (4.5μm) than conventional latex beads (0.75μm), and are clearly labelled, which allows individual beads to be distinguished and ensures rapid quantification of uptake. Furthermore, direct visualisation of cells allows various uptake parameters to be quantified and correlated directly with DC morphology.

5.2 Results

5.2.1 Effect of MAPK inhibitors on DC morphology

In initial control experiments, the effects of the inhibitors were investigated with respect to DC morphology. Treatment with the p38 or JNK inhibitors SB or SP had no effect on morphology. The DC resembled untreated, immature DC. Remarkably however, cells treated with the MLK inhibitor CEP had a pronounced dendritic morphology and resembled DC stimulated with LPS (figure 5.1).

Close inspection reveals that all five populations of DC shown in figure 5.1 contain two qualitatively distinct morphological populations. Firstly "round" cells that are approximately spherical with many small spiky projections, and secondly "dendritic" shaped cells, which are more extended and are characterised by long dendritic projections.

In the untreated, SB and SP groups, the vast majority of DC are round while a small proportion are dendritic shaped. However, upon treatment with LPS or CEP this equilibrium of morphologies is altered and the dendritic shaped cells become the predominant population, while a minority of cells remain round.

These observations suggest that MLK is involved in controlling DC morphology, and more specifically, that MLK activity biases the DC towards the rounded and "immature" morphology. However, this property of MLK appears to be independent of its MAP3K role upstream of p38 and JNK, since inhibiting these enzymes has no effect on DC morphology. To investigate whether this was a concentration-dependent effect, DC were treated with a titration of each inhibitor. The number of dendritic- and round-shaped cells in each field was quantified and the proportion of dendritic shaped cells calculated (figure 5.2). Even at the highest concentrations, SB and SP did not induce an increase in the proportion of dendritic shaped cells, indicating that the ability of MLK to induce morphological alteration of DC is a MAPK-independent function.

5.2.2 Effect of MLK inhibition on endocytic capacity of DC

In the next experiments the effect of the MLK and MAPK inhibitors was investigated with respect to the ability of DC to internalise FITC-conjugated dextran (figure 5.3). FN-adhered DC were studied; dextran was added to the adherent DC, and then following appropriate stimulation, the cells were de-adhered by trypsinisation and analysed by flow cytometry.

In parallel to the morphological analysis, the SB and SP inhibitors did not alter the endocytic capacity of DC. In contrast, the endocytic capacity of CEP-treated DC was reduced to a level identical to that of LPS-treated cells.

CEP- and LPS-treated DC demonstrate both an increased proportion of dendritic-shaped cells and a reduced capacity to internalise FITC-conjugated dextran. We therefore hypothesised that these two phenomena are linked; induction of dendritic-shaped cells resulting in loss of antigen-internalisation capacity. We could not investigate this hypothesis however using current techniques, and therefore we were required to design a novel assay.

5.2.3 Optimisation of bead internalisation

Preliminary studies examined the binding of uncoated microbeads. These microbeads were considered to be an ideal tool to investigate the antigen uptake capacity of DC due to the ease with which DC internalised the beads, the ideal size of the beads relative to the DC and their visibility under the microscope. However, DC bound uncoated beads inefficiently and, as a result, the proportion of beads internalised was low. The beads were therefore targeted to the DC using the cell surface receptor, DC-SIGN (Geijtenbeek et al., 2000). This C-type lectin was chosen due to its specificity for the cell type, its high and relatively constant level of expression upon maturation and its role in binding and internalisation of viruses and bacteria (van Kooyk and Geijtenbeek, 2003; Steinman, 2000). A diagram of the experimental protocol is shown in figure 5.4 and examples of images obtained in figure 5.5.

5.2.4 Optimisation of microbead targeting to DC

Initial experiments confirmed the stable expression of DC-SIGN on DC, both with and without LPS (figure 5.6).

To analyse and quantify the microbead experiments, six parameters were calculated for each field; the total number of DC, the number of dendritic-shaped DC, the number of DC containing one or more microbeads, the total number of microbeads in the field, the number of cell-associated (internal and external) microbeads, and the number of internal microbeads. From these raw data, the ability of DC to bind microbeads was examined by calculating the percentage of microbeads that are cell associated. Three parameters were calculated to analyse the capacity of DC to internalise microbeads; the percentage of DC with one or more internalised microbeads, the mean number of beads internalised per cell in the field, and the percentage of the total cell associated microbeads that are internal (section 2.9.6).

We first examined the ability of anti-DC-SIGN antibodies to target microbeads to DC, relative to an isotype control antibody (figure 5.7). As expected, DC-SIGN targeting enhanced both the percentage of total beads that were cell associated (figure 5.7a), and the proportion of cells that had taken up beads (figure 5.7b) significantly. Interestingly, although the mean number of beads taken up per cell was far greater in the presence of DC-SIGN antibody (figure 5.7c), the percentage of cell-associated beads that were internalised was identical for DC-SIGN and isotype-labelled beads (figure 5.7d). DC-SIGN targeting therefore enhanced binding of microbeads to the DC surface, but not internalisation.

To optimise the targeting of microbeads further, PE-conjugated anti-DC-SIGN antibodies were titrated and the ability of DC to bind and phagocytose the resulting beads was quantified. As expected, the percentage of beads that were cell-associated increased in proportion to the concentration of targeting antibody (figure 5.8a). This correlation was also observed in the mean number of beads internalised per cell (figure 5.8b). The proportion of cells containing one or more beads also increased with antibody concentration (figure 5.8c), although even the lowest concentration of targeting antibody used was sufficient to induce bead internalisation in greater than 75% of the cells. Strikingly, as noted in figure 5.7d, the percentage of cell-associated beads internalised was independent of antibody concentration (figure 5.8d).

5.2.5 Effects of LPS treatment on phagocytic capacity and dendrite pattern of DC

LPS is a well-characterised TLR4 ligand that induces DC maturation (Ardeshna et al., 2000; De Smedt et al., 1996) and has been shown to induce downregulation of antigen uptake by DC. Therefore LPS-treated DC were used to explore microbead internalisation further in a quantitative fashion.

To determine the optimum period of incubation of cells with beads we examined the ability of control versus LPS-treated DC to internalise beads over time. Three parameters of microbead uptake capacity were recorded; the mean number of beads internalised per cell (figure 5.9a), the percentage of cells containing beads (figure 5.9b), and the ability of cells to internalise cell-associated beads (figure 5.9c). All three techniques yielded a remarkably similar pattern of microbead internalisation. In each case control DC were approximately twice as proficient at microbead uptake compared to LPS-treated at any given time-point.

The effects of LPS concentration and temperature on microbead uptake by DC were analysed (figures 5.10a-c). All three measures of microbead uptake capacity showed a dramatic downregulation of internalisation in response to LPS treatment.

To validate bead internalisation as a measure of the capacity of DC to internalise antigen, the ability of control and LPS-treated DC to internalise microbeads also was compared to the more established technique of FITC-conjugated dextran uptake. FITC-conjugated dextran is known to bind to the mannose receptor, a lectin that is related to DC-SIGN. Analysis of uptake of FITC-conjugated dextran by DC reveals a dose dependent decrease in dextran internalisation with increasing LPS concentration (figure 5.10d).

The downregulation demonstrated by all three assays of microbead internalisation was more striking than that measured by FITC-conjugated dextran uptake (figure 5.10a-c versus 5.10d). As observed for microbead internalisation, incubation at 4°C completely abrogates FITC-conjugated dextran uptake (figure 5.10d).

5.2.6 Correlation between dendritic morphology and internalisation capacity of DC

The quantification of antigen uptake by direct visualisation of cells allows the examination of the relationship between morphology and internalisation capacity. Therefore, in parallel to examining microbead uptake, the effect of LPS on the morphology of DC adhered to FN was studied. LPS induced an increase in the proportion of dendritic-shaped cells (defined as cells possessing dendritic projections of greater than twice the cell body in length) (figure 5.11.1).

Since LPS induces an increase in dendritic morphology (figure 5.11.1) in parallel to a downregulation of uptake capacity (figure 5.10), we hypothesised that dendritic-shaped DC are less proficient at internalising microbeads compared to round cells. Therefore the relative ability of round versus dendritic shaped cells to engulf microbeads was examined. A ten-minute incubation period was used in contrast to the ninety minutes used in other experiments because DC are known to be dynamic in shape and may alter their morphology between round and dendritic. In both control and LPS-treated groups, round cells were more proficient at internalising microbeads than those that had a pronounced dendritic appearance, and this difference was significant (figure 5.11.2), indicating that the shape of the cells is an important determinant of the ability of DC to internalise microbeads.

5.2.7 Use of alternative antibodies to target microbeads to DC

Given that the percentage of cell-associated beads internalised appeared to be independent of the avidity of DC for the microbeads, (figures 5.7d and 5.8d), we investigated whether microbead targeting to receptors other than DC-SIGN would give equivalent phagocytic efficiency. In particular, we wondered whether the levels of receptors whose expression are susceptible to LPS-induced regulation correlate to uptake efficiency.

The surface marker proteins CD80, CD83, and MHC Class I were selected as prototypic LPS-regulated molecules (figure 3.2a), while the pseudo-MHC Class I molecule CD1a (figure 3.1b), and DC-SIGN (figure 5.6) were tested as receptors whose level of expression is independent of LPS treatment. Strikingly, analysis of the percentage of cell-associated beads internalised indicates a consistent reduction of around 50% in uptake by LPS-treated DC relative to untreated, irrespective of the surface molecule targeted by the bead.

5.2.8 Effects of CEP treatment on microbead uptake capacity of DC

Having designed an assay to investigate the correlation between the morphology and antigen uptake capacity of DC, and examined the robustness of this assay, we wished to use this strategy to investigate the effects of CEP treatment on DC.

In figures 5.1 and 5.2, it was shown that CEP induces an increase in the proportion of dendritic-shaped DC, identical to the morphological effects of LPS treatment. Incubation with CEP or LPS also reduced the capacity of DC to internalise FITC-conjugated dextran (figure 5.3). It was subsequently shown that in response to LPS treatment, the morphology and antigen uptake capacity of DC are linked and reduction in antigen internalisation by LPS is in fact a consequence of the increase in the proportion of DC with dendritic morphology (figure 5.11.2). Analogous to this, as shown in figure 5.13, treatment of DC with CEP (or LPS as a positive control) had no effect on the underlying ability of the DC to internalise antigen relative to control-treated DC.

In an identical manner to LPS, therefore, data presented in this chapter suggests that the ability of CEP to downregulate antigen internalisation (figure 5.3) is an indirect phenomenon occurring as a result of the ability of CEP to increase the proportion of dendritic-shaped DC.

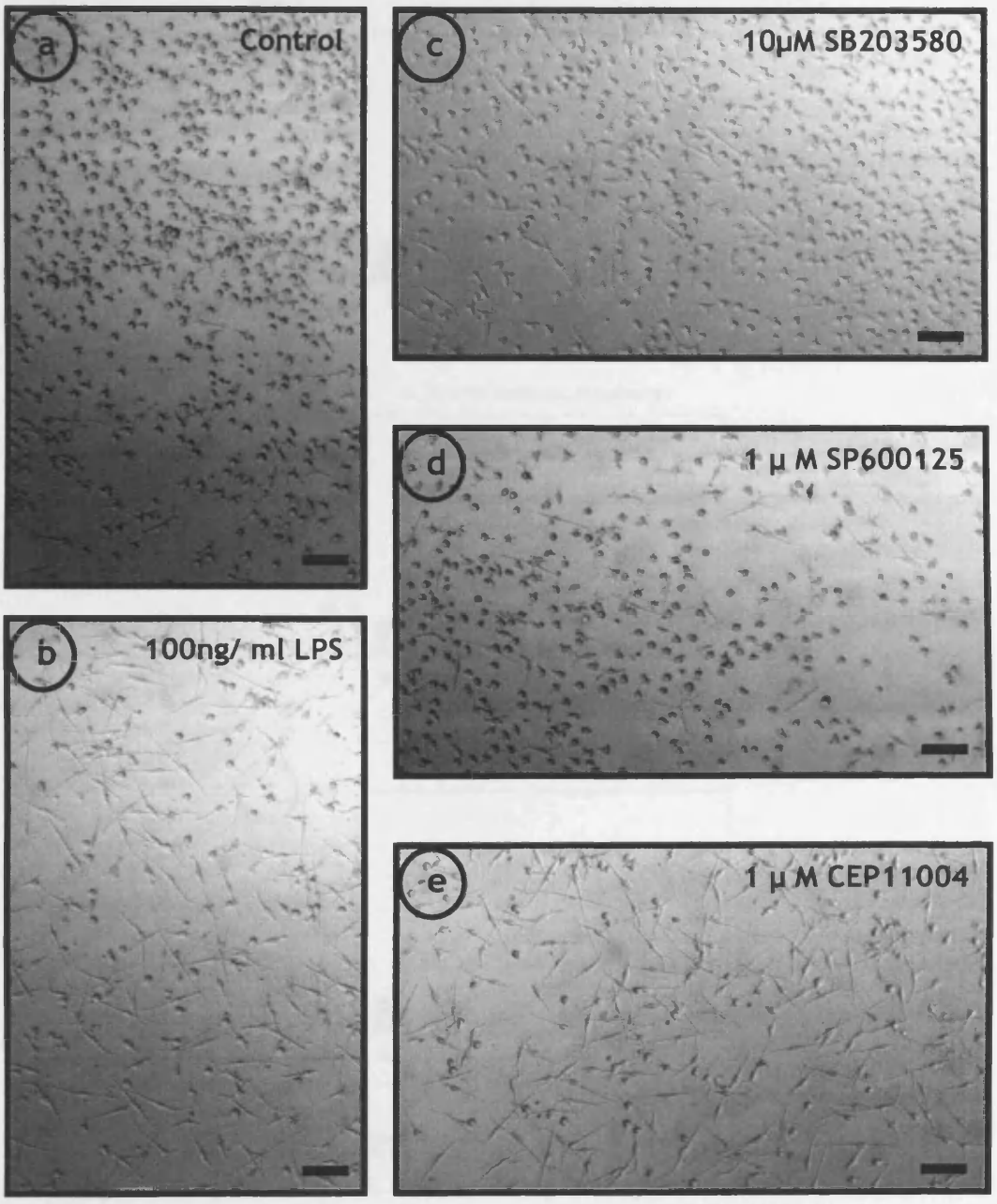

Figure 5.1. Effect of MAPK pathway inhibitors on DC morphology

Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of: (a) no stimulus, (b) 100ng/ml LPS, (c) 10μM SB203580, (d) 1μM SP600125, (e) 1μM CEP11004. They were then incubated for a further 18hrs prior to examination by confocal microscopy. Representative of three individual experiments is shown. Scale bar represents 50 μm.

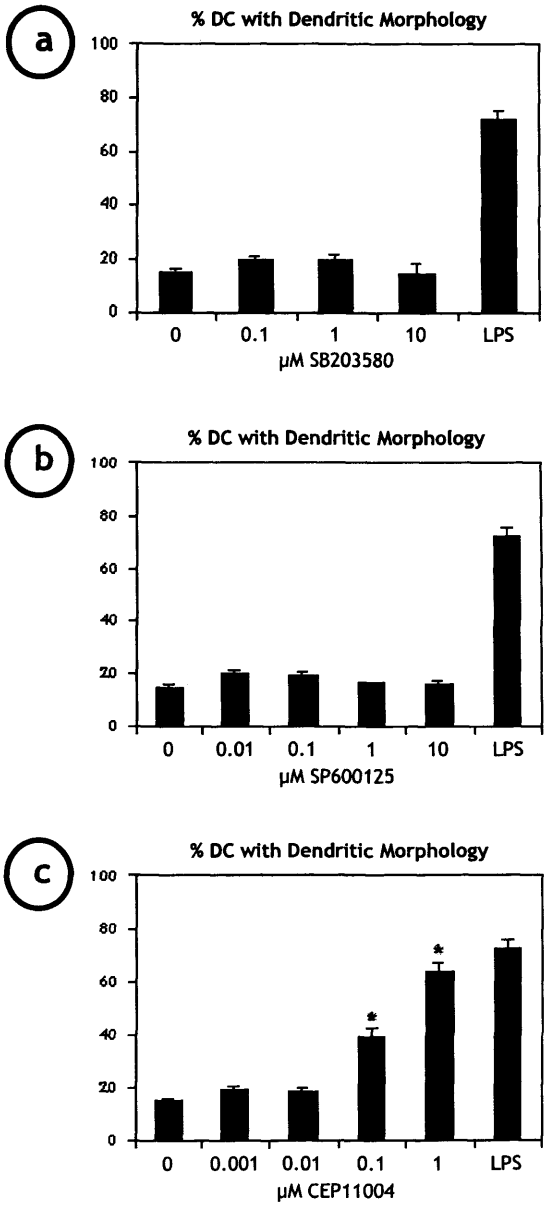

Figure 5.2. Quantification of the effect of MAPK pathway inhibitors on DC morphology

Images of DC were were obtained as described in figure 5.1. The percentages of dendritic shaped cells were then calculated. "Dendritic shaped cells" defined as possessing at least one dendritic process of greater than twice the cell body in length. Mean of three individual experiments. Error bars indicate standard error of the mean, and asterisks indicate $p<0.05$ versus untreated control.

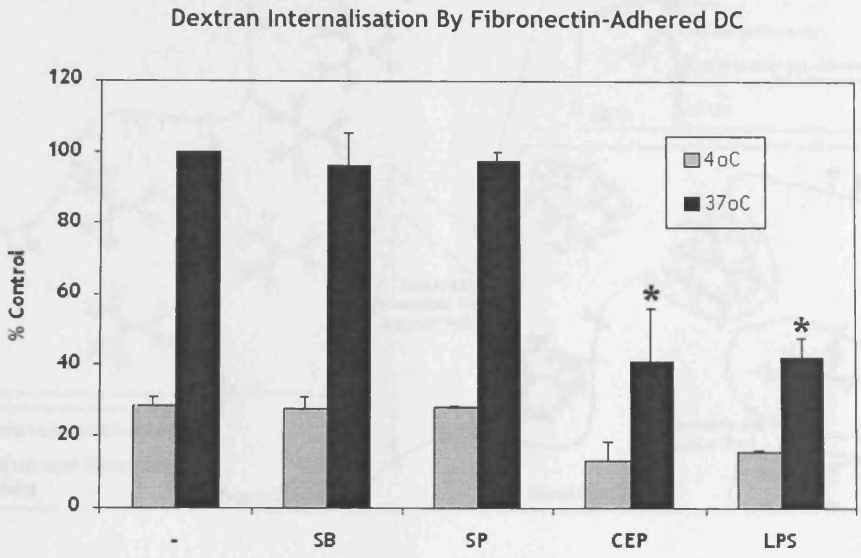

Figure 5.3. Uptake of FITC-conjugated dextran by LPS and inhibitor-treated DC

Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of 10μM SB203580, 1μM SP600125, 1μM CEP11004, or 1000ng/ml LPS as indicated and incubation for a further 18hrs. FITC-conjugated dextran was then added to a final concentration of 30μg/ml for 90 minutes at 37°C or 4°C as indicated. Cells were then de-adhered by trypsinisation and washed before analysis by flow cytometry. Mean of three individual experiments is shown. Error bars indicate standard error of the mean, and asterisks indicate $p<0.05$ versus untreated control (calculated from raw median fluorescent intensity values).

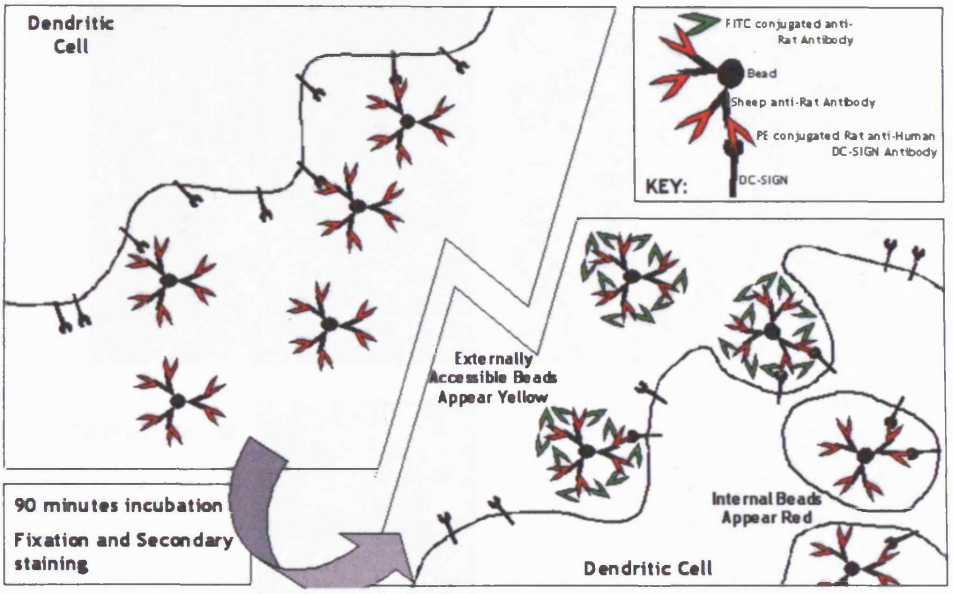

Figure 5.4. Experimental procedure to quantify antigen uptake capacity of adherent cells

Diagram outlining experimental protocol. Adherent DC are incubated with PE-labelled anti-human-DC-SIGN rat antibody coated microbeads for 90 minutes. They are then fixed and stained with FITC-labelled polyclonal anti-rat antibodies. Internalised microbeads appear red, whereas microbeads accessible to the extracellular milieu fluoresce in both red and green channels and thus appear yellow.

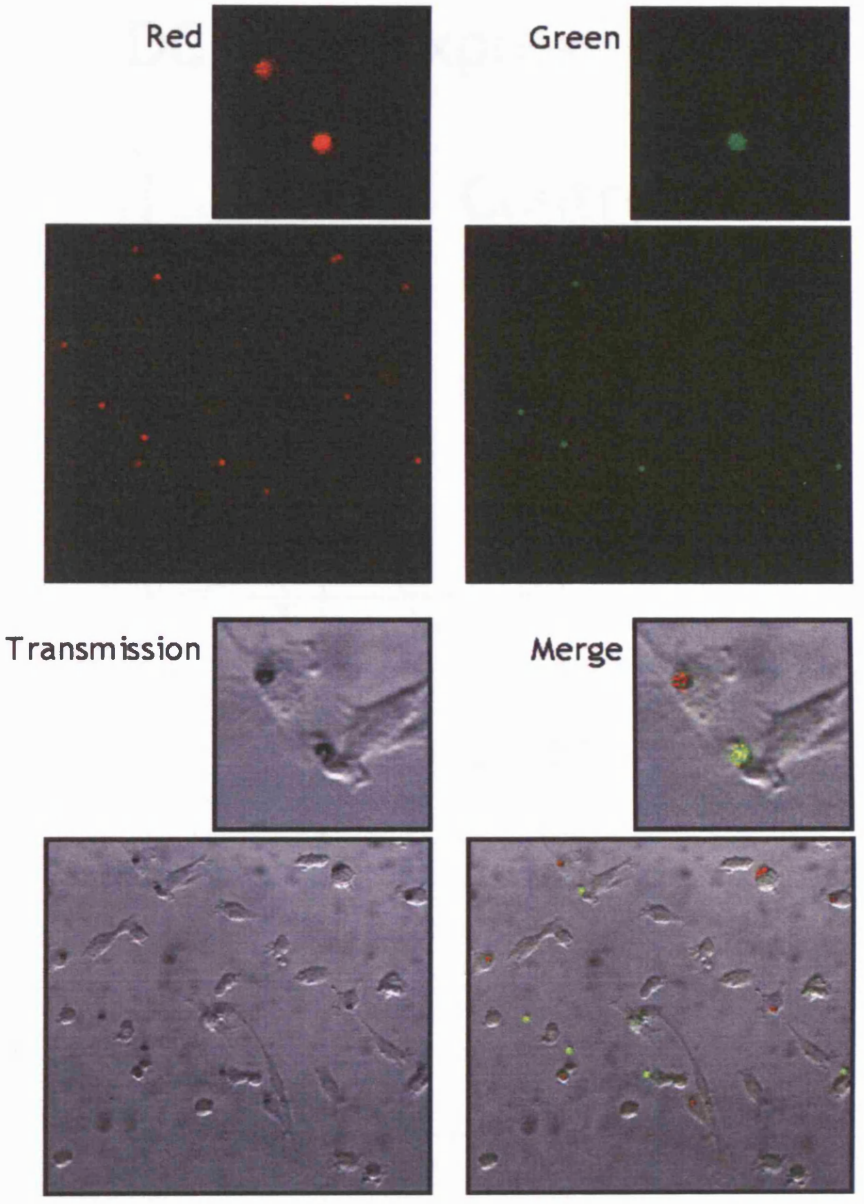

Figure 5.5. Example of images produced for quantification of microbead uptake

Internalised microbeads appear red, whereas externally accessible microbeads are also stained with FITC-conjugated antibody, thus appear yellow.

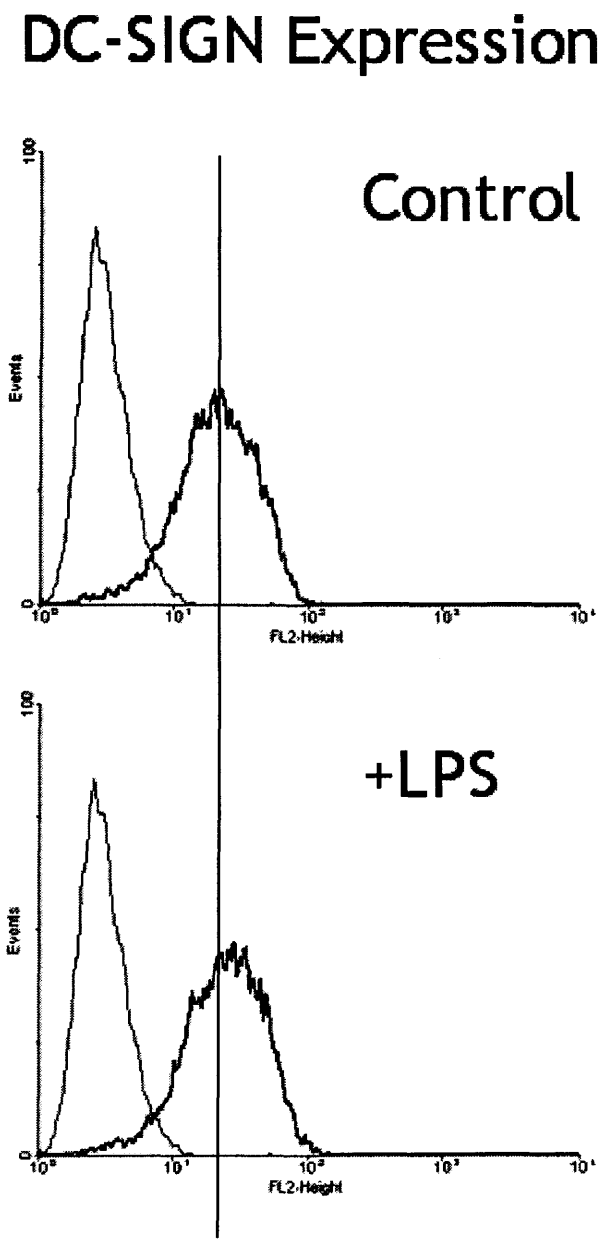

Figure 5.6. DC-SIGN Expression in DC

Immature DC were incubated with or without 1000ng/ml LPS for 18 hours prior to analysis of DC-SIGN expression by flow cytometry. One representative of three experiments is shown.

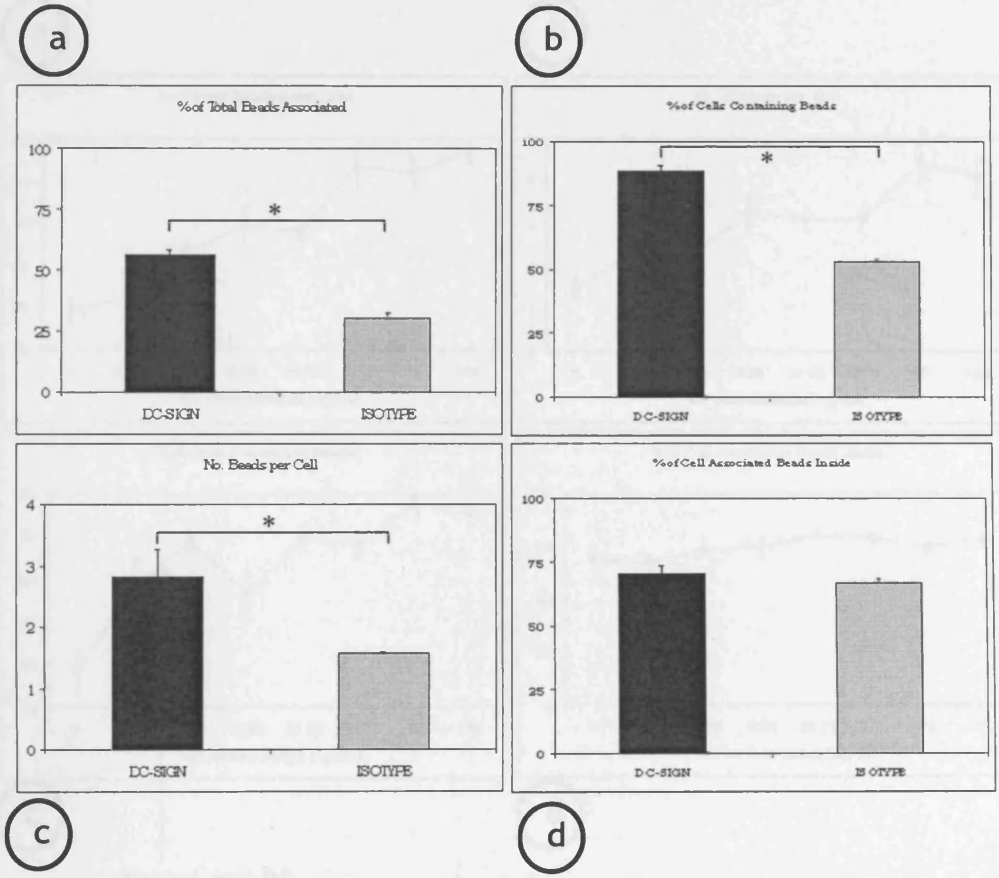

Figure 5.7. Quantification of microbead internalisation capacity using DC-SIGN targeted versus non-targeted (isotype matched control antibody-coated) microbeads

Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of microbeads and further incubation for 90 minutes before fixation and staining. Representative of three individual experiments is shown. Error bars indicate standard error of the mean, and asterisks indicate $p<0.05$.

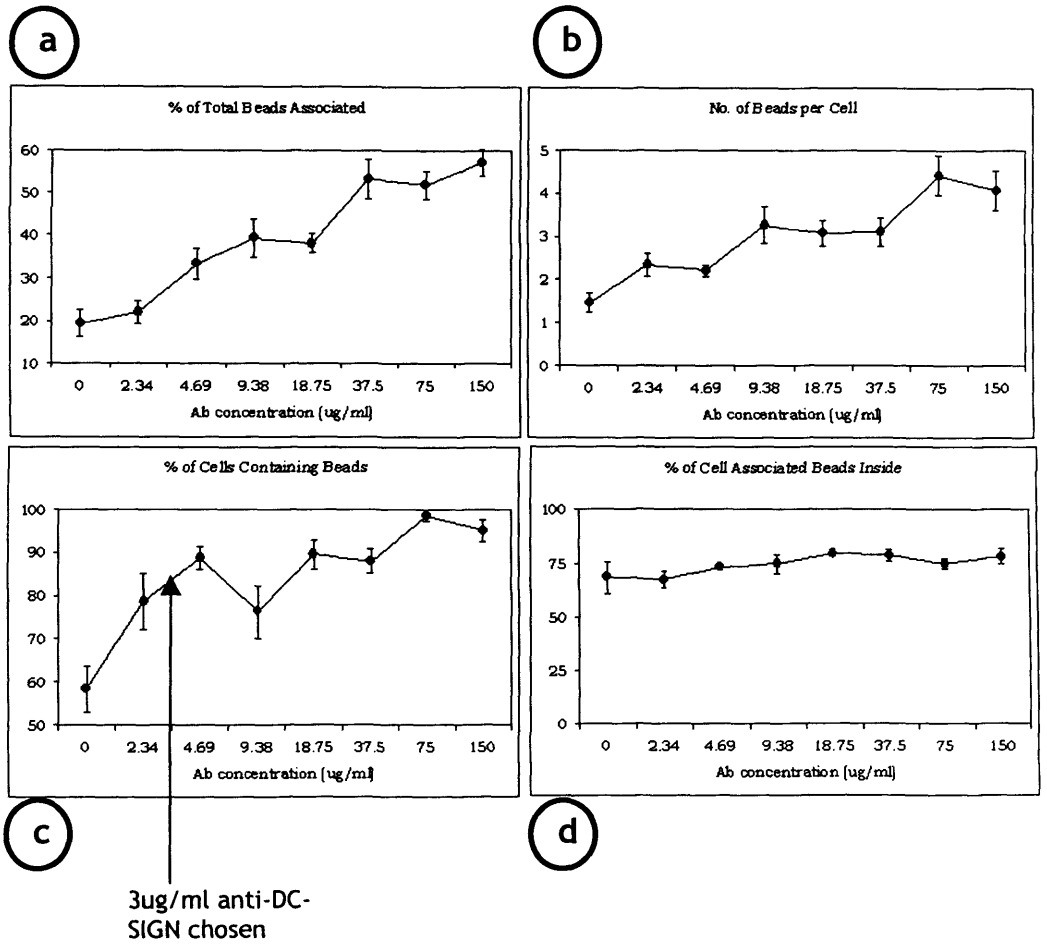

Figure 5.8. Comparison of cell binding and internalisation of microbeads coated with different anti-DC-SIGN antibody concentrations

Indicated are the antibody concentrations in which 1 x 10^6 microbeads were incubated during antibody coating. Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of microbeads and further incubation for 90 minutes before fixation and staining. Representative of three individual experiments is shown. Error bars indicate standard error of the mean.

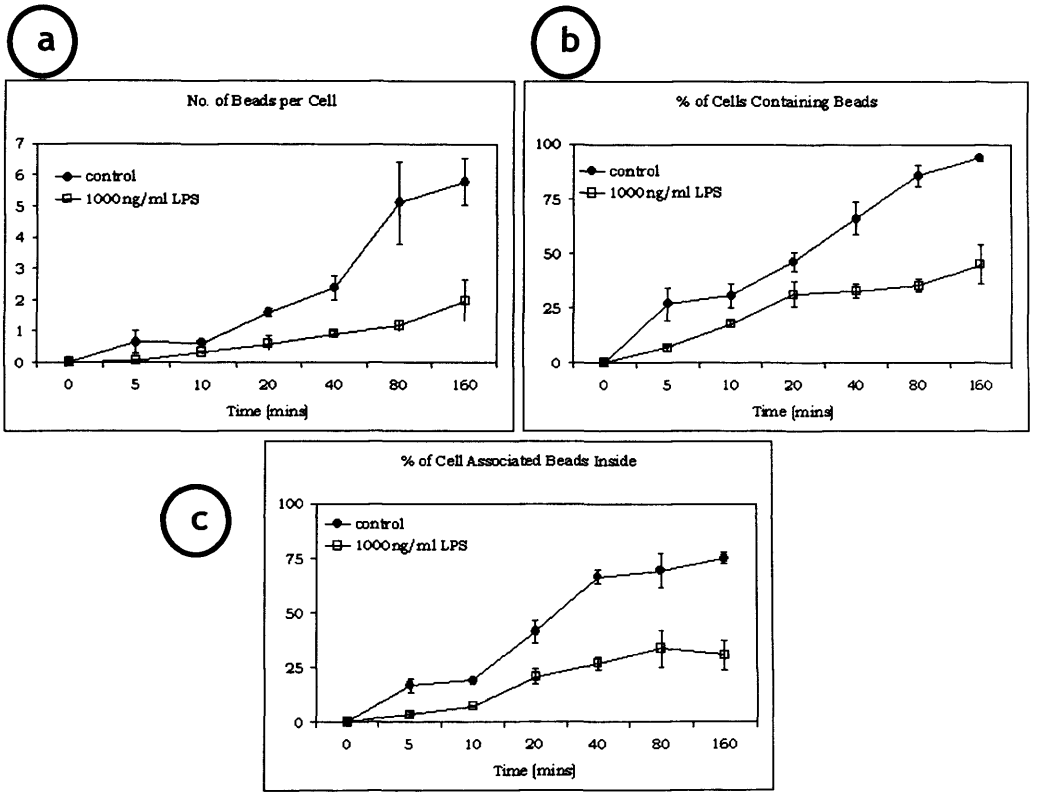

Figure 5.9. Ability of control versus LPS-treated DC to internalise microbeads over time

Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of 1000ng/ml LPS as indicated and incubation for a further 18hrs. DC-SIGN-coated microbeads were then added and cells incubated for a further period as indicated before fixation and staining. Representative of three individual experiments is shown. Error bars indicate standard error of the mean.

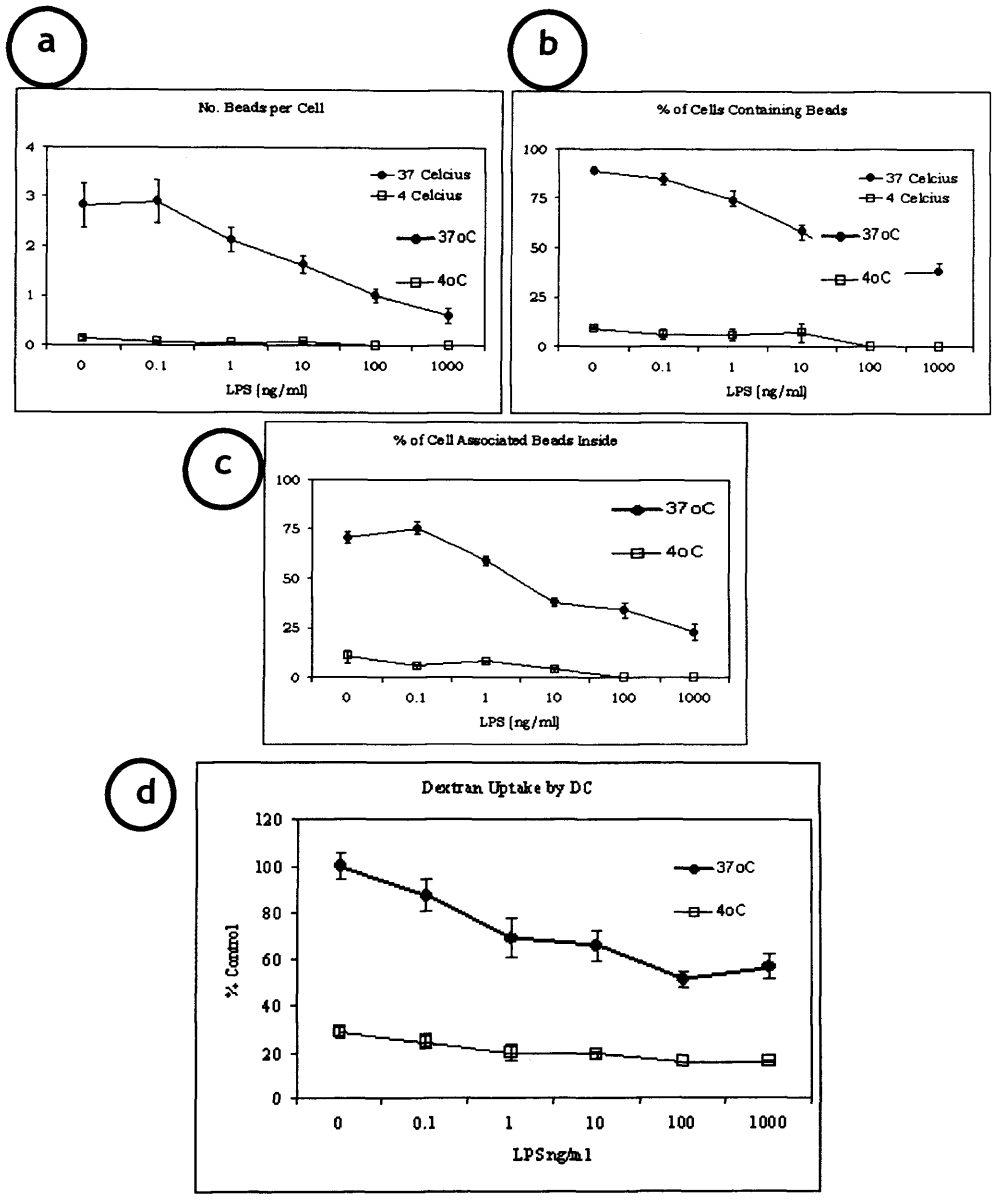

Figure 5.10. Analysis of antigen uptake capacity of control versus LPS-treated DC

Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of LPS as indicated and incubation for a further 18hrs. Antigen uptake capacity was then assessed by internalisation of DC-SIGN targeted microbeads (a-c) or FITC-conjugated Dextran (d). (a-c) Microbeads were added and cells incubated for a further 90 minutes at 37°C or 4°C as indicated before fixation and staining. Representative of three individual experiments is shown. Error bars indicate standard error of the mean. (d) FITC-conjugated dextran was added to a final concentration of 30μg/ml for 90 minutes at 37°C or 4°C as indicated. Cells were then de-adhered by trypsinisation and washed before analysis by flow cytometry. Mean of three individual experiments is shown.

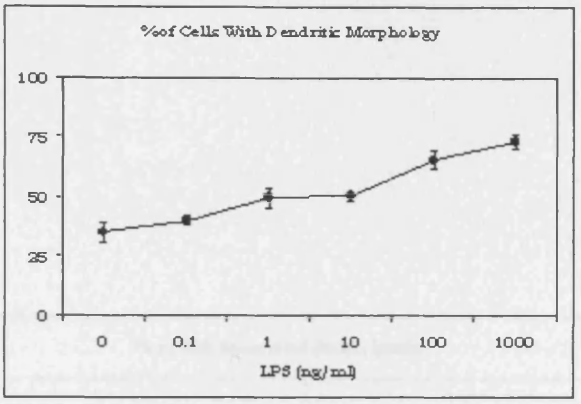

Figure 5.11.1. Morphological analysis of LPS-treated DC

Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of LPS as indicated and incubation for a further 18hrs. Representative of three individual experiments is shown. Error bars indicate standard error of the mean.

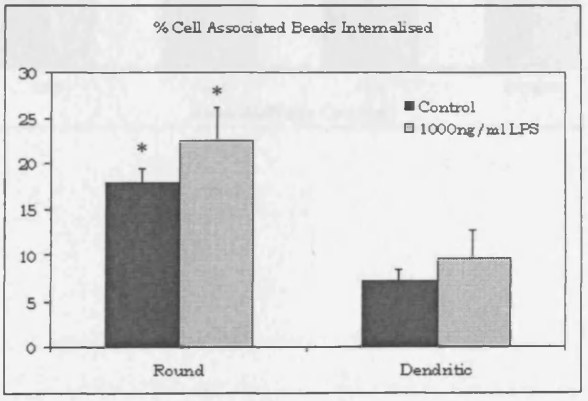

Figure 5.11.2. Ability of round versus dendritic DC to internalise microbeads

Cells were incubated on FN-coated glass coverslips overnight, prior to addition of 1000ng/ml LPS as indicated and incubation for a further 18hrs. Microbeads were then added and cells incubated for a further 10 minutes before fixation and staining. Indicated is the percentage of cell-associated beads internalised for the round and dendritic cells in each field and expressed with the standard error of the mean. Mean of three individual experiments is shown. Asterisks indicate $p<0.05$ versus equivalently treated dendritic cells.

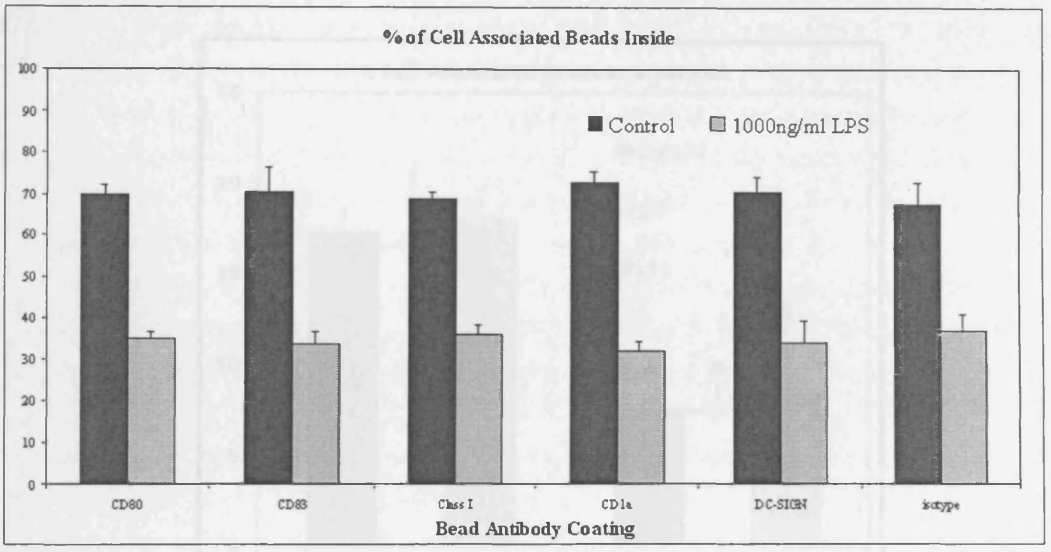

Figure 5.12. Internalisation of microbeads targeted to alternative surface marker proteins

Analysis of the ability of DC to internalise bound microbeads targeted to various surface proteins as indicated. Immature DC were incubated on FN-coated glass coverslips overnight, prior to addition of 1000ng/ml LPS as indicated and incubation for a further 18hrs. Microbeads were then added and cells incubated for a further 90 minutes before fixation and staining. Mean of three or five (DC-SIGN group) individual experiments is shown. Error bars indicate standard error of the mean.

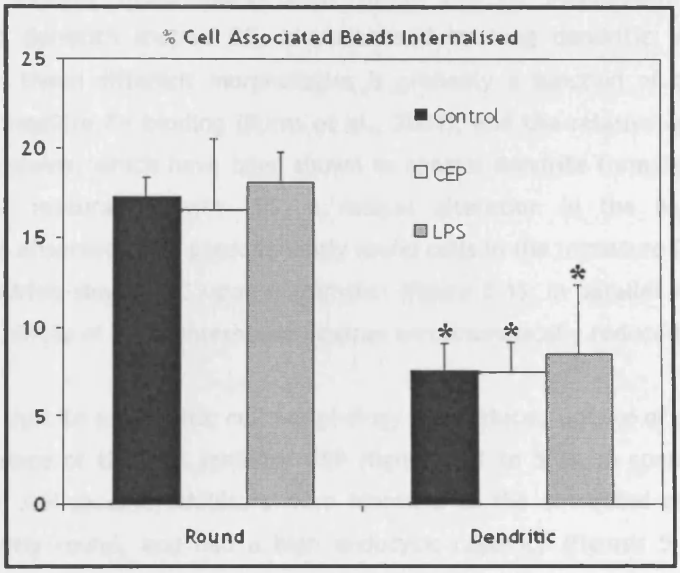

Figure 5.13. Ability of round versus dendritic DC to internalise microbeads

Cells were incubated on FN-coated glass coverslips overnight, prior to addition of 1μM CEP11004 or 1000ng/ml LPS as indicated and incubation for a further 18hrs. Microbeads were then added and cells incubated for a further 10 minutes before fixation and staining. Indicated is the percentage of cell-associated beads internalised for the round and dendritic cells in each field and expressed with the standard error of the mean. Mean of at least three individual experiments is shown. Error bars indicate standard error of the mean, and asterisks indicate $p<0.05$ versus untreated control.

5.3 Discussion

DCs *in vivo*, such as LCs, exhibit a more dendritic morphology than DC observed *in vitro*. This is probably due to an increased availability of ligands (in three dimensions) to which the cells can anchor. Indeed the use of FN as a substratum induces an *in vitro* scenario which may be more like that seen *in vivo* when compared to that seen when DC adhered to plastic alone, presumably due to the increased ability of the cells to adhere (Swetman et al., 2002). Using a FN substratum to model the *in vivo* environment, we have observed that despite phenotypic uniformity, two cellular morphologies exist within the DC population: a spiky round morphology, and dendritic-shaped DC, characterised by long dendritic projections. The balance between these different morphologies is probably a function of expression of β_1 integrins, which mediate FN binding (Burns et al., 2004), and the relative activities of Rho, rac and cdc42 proteins, which have been shown to control dendrite formation (Swetman et al., 2002). Upon maturation with LPS, a radical alteration in the balance of these morphologies was observed from predominantly round cells in the immature DC population to an excess of dendritic-shaped DC upon maturation (figure 5.1). In parallel with this shift in morphology, the ability of DC to internalise dextran was dramatically reduced (figure 5.3).

Unexpectedly, a shift to a dendritic cell morphology and reduced uptake of dextran was also seen in the presence of the MLK inhibitor CEP (figures 5.1 to 5.3). In contrast, DC treated with the p38- or JNK-specific inhibitors were identical to the untreated cells in that they were predominantly round, and had a high endocytic capacity (figures 5.1 to 5.3). The effects of MLK inhibition on morphology (in contrast to the effects on DC maturation) were therefore not mediated via the downstream activity of MAPKs. Importantly, the CEP-treated DC are not mature in terms of surface phenotype (figure 4.7), and indeed are unable to mature phenotypically when treated with maturation stimuli such as LPS (figure 4.8). This suggests that MLK proteins are involved in controlling DC morphology and antigen uptake via a mechanism that is independent of MAPKs. Evidence for possible mechanisms of MAPK-independent regulation of cellular morphology by MLK proteins will be discussed in Chapter 6. However, CEP is therefore a tool with which the phenotypic and morphological aspects of DC maturation can be separated - offering an exciting novel avenue for future investigation.

Studies correlating morphological parameters and antigen uptake capacity of DC have not previously been published. To measure the antigen internalisation capacity of DC under conditions where they retain their characteristic morphology, we have had to overcome several technical issues. Current methods of analysing antigen uptake by DC involve the internalisation of fluorescently labelled molecules such as dextran, which is then quantified by flow cytometric or fluorescent microscopic methods. Obviously flow cytometric

measurement uses non-adherent cells, preventing the concurrent analysis of cellular morphology. Many microscopic techniques, on the other hand, also have drawbacks. For example, it is difficult to conclusively distinguish between internal and externally bound probe. The method outlined in this chapter facilitates simple distinction between internal and external beads without complex washing or quenching steps, while maintaining the ability to undertake parallel morphological analyses. In addition, microbeads are easily visualised and thus quantified, and provide stable, non-degradable probes for internalisation.

The mechanism by which the microbeads are internalised has not been investigated in detail in this study. *In vivo*, DC are known to internalise antigen via several mechanisms (section 1.1.3.1), including both receptor-ligation-induced, and non-specific uptake systems. Initially, beads were targeted to DC-SIGN, which is a C-type lectin related to the mannose receptor, and has been shown to function as an endocytic receptor (Guo et al., 2004). As expected, the anti-DC-SIGN antibody greatly enhanced the number of DC-associated beads and the proportion of DC which had bound microbeads. However, it does not appear that the internalization of anti-DC-SIGN-coated microbead requires specific receptor-induced signals, since the internalisation of DC-associated DC-SIGN-coated microbeads, as a proportion of cell associated beads, is no more efficient than the uptake of isotype control antibody-coated microbeads (see Figs 5.7d, 5.8d, and 5.12). Since DC take up microbeads independently of the mechanism by which they are bound to the cell, internalisation of cell-associated microbeads may occur as a result of membrane invagination during macropinocytosis engulfing the cell-associated microbead. In support of this hypothesis, macropinosomes are known to be much larger than vesicles formed as a result of other forms of uptake such as clathrin-associated endocytosis, or lipid raft-mediated uptake (Johannes and Lamaze, 2002). Macropinosomes are known to be of the same approximate size as the microbeads, suggesting that uptake of volumes of this size is a physiologically relevant phenomenon. Indeed, we have observed cells (including dendritic-shaped cells) containing in excess of 20 microbeads upon longer incubation periods and at high bead densities (data not shown), illustrating that the size of the microbeads is not a limiting factor in their internalisation. Inhibition of PI3K using wortmannin has been shown to inhibit the formation of macropinosomes by DC (West et al., 2000), so it would be interesting to investigate whether this treatment prevents microbead internalisation. The receptor-independence of microbead internalisation is therefore a further strength of the assay, which is not dependent upon a particular receptor or mechanism of uptake, but rather reflects the inherent capacity of the cells to internalise antigen. Analysis of targeted microbeads therefore provides a novel and useful way to approach dynamic structure-function relationships that has not been possible in previous DC studies.

Microbead uptake was quantified in terms of three main parameters, the mean number of beads internalised per cell, the percentage of cells containing one or more internalised beads, and the percentage of cell-associated beads that have been internalised. The first two measures showed similar patterns of relative uptake capacities between groups (figure 5.7b versus 5.7c, figure 5.8b versus 5.8c, figure 5.9b versus 5.9c, and figure 5.10b versus 5.10c). The agreement between these two parameters supports the interpretation that the changes seen reflected the population of DC as a whole, rather than very large changes in a very small proportion of cells. In immature unstimulated DC populations, one can envisage that *most* cells possess a *high* antigen uptake capacity, while some possess a *low* antigen uptake capacity. Upon maturation with LPS, the balance is shifted, and *most* cells possess a *low* capacity to uptake (figures 5.9 and 5.10). This equilibrium in microbead internalisation capacity of both immature and mature DC is reminiscent of the balance of round and dendritic DC morphologies, whereby in the unstimulated population, *most* cells are round, and, following maturation, *most* cells have dendritic morphologies (figures 5.1 and 5.2). Indeed, the down-regulation of the internalisation capacity of DC (figure 5.10), and the increase in the proportion of DC with a dendritic morphology (figure 5.11.1), both closely correlate with the concentration of LPS. The novel phagocytic assay described here permitted us to test the relationship between uptake and morphology at a single cell level.

Examining the ability of round and dendritic shaped immature DC to engulf microbeads indeed confirmed that round cells were significantly better at internalising cell-associated microbeads (figure 5.11.2), suggesting the possibility that the ability of LPS to induce dendrite formation and to reduce antigen uptake may be related. Indeed, the data presented here indicate that the reduction in microbead uptake observed following LPS treatment is a function of the change in the proportion of the different shaped cells; from a population of predominantly round cells (with high phagocytic capacity) to a vast majority of dendritic shaped cells (with a reduced phagocytic capacity). At a single cell level, LPS has little effect on uptake by either round or dendritic shaped cells, and may even enhance this property slightly for both cell morphologies (figure 5.11.2). An objection to this interpretation might be that dendritic shaped cells in the immature population may represent a small contaminating population of DC that have become matured (and therefore show low uptake). Likewise, the round cells in the mature population may be DC that have remained immature despite LPS treatment (and therefore retain high uptake). When examined by time-lapse microscopy, DC *in vitro* can be observed to continually extend and retract dendrites as they randomly migrate and thus alternate between apparent round and dendritic morphologies (Swetman et al., 2002). Time-lapse microscopy of cells during bead uptake would confirm

whether the avidly phagocytic cells are indeed permanently "immature", or simply reflect the round morphology at the particular instant at which they take up beads.

The mechanism underlying why round cells phagocytose more efficiently than dendritic cells remains unknown, and requires further investigation. We would like to propose a hypothesis, however, that seems to account for all of the data presented in this chapter. The formation of the relatively large vesicles required to encapsulate the microbeads presumably requires the rapid commitment of a significant proportion of the cell membrane. A very fundamental reason why round DC take up antigen more efficiently than dendritic shaped DC may therefore be their smaller surface area to volume ratio, which results in their having more 'spare' membrane with which to invaginate and internalise antigens. The reduced uptake by LPS treated population may therefore directly result from altered morphology of most of the cells, rather than reflecting specific changes in the biochemical steps of endocytosis. In particular, this hypothesis is attractive because it could explain why CEP inhibitor-treated cells, although showing an immature phenotype, nevertheless show reduced bead uptake, reflecting their very enhanced dendritic morphology.

The model of cells oscillating between different morphologies and the relationship between these morphologies and phagocytic capacities may also apply *in vivo*. This is indeed supported by the observation that Langerhans cells endocytose substrates such as FITC-conjugated dextran with a much lower efficiency than immature DC in culture (Steinman et al., 1999), and furthermore, macrophages, which resemble round DC, phagocytose *in vitro* with a greater capacity than DC (Kiama et al., 2001; Thiele et al., 2001). Upon receipt of a maturation stimulus *in vivo*, DC must de-adhere and adopt a round morphology as they begin a program of migration to the lymph nodes. It is attractive to speculate that during this round phase, DC possess an increased capacity to phagocytose in an attempt to maximise their antigen load prior to departing the infected area. This would correlate with the observations that TLR or CCR7 ligation on DC results in a transient increase in endocytic capacity *in vitro* (Blander and Medzhitov, 2004; Yanagawa and Onoe, 2003).

The morphology of DC is unique, in showing very long cellular processes (more neuronal-like than monocyte/macrophage like), but the dendrites are short-lived and highly dynamic structures. Using two photon imaging to visualise DC and lymphocytes in an *ex vivo* lymph node, Miller et al observed that mature DC extended long agile dendrites and these projections were the site of T cell interaction. The possession of dendrites therefore increases the surface area of DC, and indeed, an individual DC was observed to be capable of interaction with up to 5000 T cells per hour. The dynamic nature of the dendrites also

increased the ability of DC to find and interact with T cells (Miller et al., 2004). It is therefore reasonable to speculate that in an analogous fashion, the possession of dendrites by DC in the periphery increases the surface area of these cells, optimising antigen detection. The model proposed in this chapter suggests that this unusual morphology may be required to allow DC to perform their function in an optimal way. The presence of long cellular processes greatly increases the overall area that is efficiently scanned for antigen or invading microbes. However, process formation may inhibit uptake, as proposed above. Rapid dynamic conversion between extended and rounded forms may thus be optimal for the sentinel function of immature DC. This model suggests that DC morphology, and in particular the flexibility of this morphology, as much as surface phenotype and cytokine secretion, may determine their remarkable efficiency in antigen processing and presentation.

Chapter 6

General Discussion

6.1 Overview

The initial objective of the study was to investigate the roles of the JNK and p38 pathway in the response of DC to oxidative stress. The hypothesis was based upon the fact that p38 is known to be involved in the control of DC maturation, and is also activated by ROS in many cell types. We therefore speculated that p38 activation by oxidative stress (modelled by H_2O_2) could influence DC maturation. Furthermore, the JNK pathway had not previously been investigated in DC, is also known to be activated by oxidative stress in other cell types, and might therefore also be involved in this process.

We found that despite activating both p38 and JNK, no DC maturation was observed in response to H_2O_2, although we did observe the induction of apoptosis, and this correlated closely with the degree of JNK activation. H_2O_2 was also incapable of modulating the ability of LPS to induce DC maturation, although a synergistic effect on both JNK activation and apoptosis was observed in response to dual stimulation with H_2O_2 and LPS, further suggesting a role for JNK in the control of DC apoptosis. The use of newly available pharmacological reagents; a JNK-specific inhibitor (SP600125), and an inhibitor of a supposedly JNK-specific MAP3K, MLK (CEP11004), confirmed a role for JNK in this process.

The inhibitors also permitted an investigation into the role of JNK in DC maturation in response to strong stimuli such as LPS, and interestingly, JNK was found not to be essential for this process. This observation was reinforced in later studies examining a greater selection of DC maturation markers and cytokines, and supported by the observation that some stimuli, which induce DC maturation, such as zymosan, do not activate JNK in DC.

Given that p38 is an important regulator of DC maturation and JNK is essential for apoptosis, it was interesting that JNK activation was consistently observed to occur at higher concentrations than p38. This suggests that there is a window of stimulation intensity in response to which DCs are induced to mature. Above this level of stimulation however, such as might be expected at highly inflammatory loci, apoptosis is induced via JNK activation. This mechanism probably serves to protect against the possibility of antigen presentation by DC that have been damaged by the highly reactive oxygen and nitrogen radicals found in the inflammatory environment.

During the course of these investigations it was observed that inhibiting MLK also prevented LPS-induced DC maturation, suggesting JNK-independent functions for this protein. A role for MLK proteins in the control of p38 activation *in vitro* had been reported, and (consistent with such a function) we observed that the MLK inhibitor prevented LPS-induced p38 activation.

This therefore introduced a second aim into the project; to investigate the role of MLK proteins in DC maturation induced by TLR ligands.

In addition to LPS, the TLR3 ligand poly (I:C), and TLR2/ TLR6 ligand zymosan were examined for their MLK requirement in the induction of DC maturation. Remarkably, the phosphorylation of p38, the upregulation of surface markers, and the secretion of cytokines in response to LPS and poly (I:C) was markedly diminished in the presence of the MLK inhibitor, whereas in contrast, zymosan-induced maturation demonstrated no requirement for MLK activity. This dichotomy in MLK dependency indicates the complexity of the regulation of DC maturation, which is crucial to the central role of these cells in the induction of highly optimised pathogen-specific immune responses.

Interestingly, upon the use of the MLK inhibitor we immediately observed a dramatic effect on the morphology of DC, a large proportion of the DC adopting an apparently mature morphology with long dendrites. In parallel to this was a concomitant reduction in the ability to internalise antigen, and using specific inhibitors we established that both of these effects were independent of p38 and JNK. We formulated a hypothesis that the two observations were linked and that the reduction in antigen uptake was due to the altered morphology of the cells. However, it was not possible to test the hypothesis using the available techniques. Therefore the design and optimisation of a novel assay permitting the parallel investigation of DC morphology and antigen uptake capacity, and the utilisation of this assay to investigate our hypothesis became a final aim of the project.

We found that DC morphology and antigen uptake were indeed linked, and that round cells possess a greater capacity to internalise antigen than extended dendritic-shaped cells. Based upon this result and the observation that DC are highly dynamic in their morphology, we suggest that this unique morphology optimises these cells for antigen processing. A dendritic phase facilitates optimal environment sensing and antigen capture, and a more rounded phase permits highly efficient uptake of bound antigen.

6.2 Activation of mixed lineage kinases

It is significant that the data presented in this thesis indicate diverse roles for MLK proteins in DC in the regulation of apoptosis, maturation, and morphology. However, there is accumulating evidence in other cell types, including the use of non-inhibitor-based approaches, to support roles for MLK proteins in these processes.

An example of this is the considerable evidence that supports a role for MLK proteins in the induction of apoptosis in other cell types (Wang et al., 2004). NGF-deprivation-induced neuronal apoptosis has been shown to proceed via rac1/cdc42-induced MLK activation (Mota et al., 2001; Harris et al., 2002; Xu et al., 2001). In fact, the MLK inhibitor used in this study was derived from a screen for compounds capable of preventing neuronal apoptosis. Studies in neuronal cell lines have indicated that the proapoptotic effects of MLK are mediated via the activation of JNK, which is known to induce apoptosis by transcription-dependent and independent mechanisms. Our data support a JNK-dependent mechanism of MLK-induced DC apoptosis in response to oxidative stress. The mechanism by which oxidative stress induces MLK activation is not clear. However, in support of the data presented in this thesis, oxidative stress-induced apoptosis of human mesencephalic neuron-derived cells has recently been demonstrated to proceed via an MLK-dependent pathway (Lotharius et al., 2005), and furthermore the antioxidant N-acetylcysteine prevents MLK3 membrane translocation and activation (Pei et al., 2005; Tian et al., 2003). We observed that treatment with H_2O_2 induces transient inhibition of tyrosine phosphatases, resulting in MLK-dependent JNK activation. This suggests that a mechanism exists by which MLKs are activated by tyrosine kinases. However, activation of MLK proteins occurs on threonine and serine residues, suggesting that this action of tyrosine kinases is probably indirect. It would be very interesting to investigate whether the activation of tyrosine kinases occurs upstream of rac and cdc42, or whether this represents an alternative mechanism of MLK activation that is independent of these GTPases.

MLK activation downstream of TLRs has not been formally reported previously, although the CEP inhibitor has been shown to prevent LPS-induced functional outcomes in other cell types (Lund et al., 2005; Ciallella et al., 2005). Consequently, the means by which TLRs induce MLK activation is not clear. There are, however, several possible mechanisms. The conventional picture of MAPK activation upon TLR ligation as outlined in sections 1.2.2.4 to 1.2.2.6 does not suggest a mechanism for MLK involvement in this process, but, several lines of evidence suggest that this picture of TLR signaling is not complete. For example, transfection of IRAK1-deficient cells with mutant IRAK proteins indicated that certain regions of IRAK1 were required for NF-κB, but not JNK activation. This suggests that these two pathways must diverge at, or upstream of, IRAK1 (Li et al., 2001). Another insight into this controversial issue of IRAK1-induced JNK activation is provided by an LPS-inducible splice variant of the adaptor protein MyD88, called MyD88s. This splice variant can bind IRAK1 but is unable to bind IRAK4. As a result there is no IRAK1 phosphorylation and consequently there is a strong inhibition of IL1-induced NF-κB activation in the presence of MyD88s, confirming the importance of IRAK1 phosphorylation in NF-κB activation (Burns et al., 2003). Interestingly, however, it has been shown that MyD88s prevents NF-κB but not AP1 reporter gene expression, and in line with this

observation, IL1-induced JNK phosphorylation is not diminished in the presence of MyD88s (Janssens et al., 2003). Clearly the precise mechanism of this signal transduction complex has not yet been fully elucidated. Mice deficient in MyD88, TIRAP, IRAK1, IRAK4 or TRAF6 have been engineered (Kawai et al., 1999; Yamamoto et al., 2002; Swantek et al., 2000; Suzuki et al., 2002; Lomaga et al., 1999). However NF-κB has been the major signaling protein of interest in these investigations, and LPS-induced activation of JNK and p38 has been investigated only in limited studies using these knockout mice.

Many signaling proteins have been suggested to be involved in TLR-mediated responses. With reference to the mechanism outlined above in which MLK proteins are activated as a result of the inhibition of tyrosine phosphatases, ROS production upon TLR4 ligation has previously been demonstrated (Ryan et al., 2004; Matsuzawa et al., 2005; Asehnoune et al., 2004; Hsu and Wen, 2002), although the mechanism of this unclear. It is therefore possible that the mechanism of MLK activation in response to TLR ligation and oxidative stress is identical. This is a particularly attractive model, since in this scenario MLK activation by oxidative stress is simply an extreme version of MLK activation by TLR ligation. ASK1 has been shown to be important for TLR signaling downstream of TLR4 in mice (Matsuzawa et al., 2005). It has been suggested that ASK1 is a critical MAP3K in the activation of JNK and p38 by ROS, both in apoptotic signaling and downstream of TLR4 (Liu and Min, 2002; Matsuzawa et al., 2005). However, we have shown that in DC, inhibition of MLK prevents H_2O_2-induced JNK activation, suggesting that ASK1 does not play a major role in MAPK activation in response to ROS in these cells. Importantly, the CEP inhibitor has been demonstrated to have no inhibitive effect on ASK1 (Harris et al., 2002; Xu et al., 2001). It is therefore possible that in human monocyte-derived DC, MLK proteins, and not ASK1, are the major MAPK activators in response to ROS, in the induction of both apoptosis and maturation.

An alternative mechanism of MLK activation by TLR ligation is suggested by the observation that the scaffold protein JIP3, which binds MLK3, MKK7, and JNK, (section 1.3.3.6) has been shown to bind to TLRs 2, 4, and 9. Obviously this suggests the existence of a single complex mediating direct signal transduction from LPS to JNK via MLK3 (Matsuguchi et al., 2003), although the pattern of TLR association does not correlate with the MLK3 dependency of TLR3 and TLR4, but not TLR2, which we observed. Furthermore, JIP3 is thought to be expressed exclusively in neurones, although expression in DC has not been investigated (Kelkar et al., 2000). MEKK1 binds JIP3 and is an important kinase in the activation of NF-κB, so it is possible that the true function of this complex is to facilitate MEKK1-mediated NF-κB activation.

In addition to the mechanism of MLK activation upon TLR ligation, the difference in MLK dependence of different TLR pathways also remains to be explained at a molecular level. One attractive hypothesis is that these differences may reflect the utilization of different intracellular adaptor proteins by the TLRs. Analysis of mice deficient for different adaptor proteins indicates that TLR3 and TLR4, but not TLR2, possesses a strong signaling requirement for the TIR-domain-containing adaptor inducing IFN-β (TRIF) protein (Yamamoto et al., 2003) (section 1.2.2.4). The observed effects are similar to those reported here in that CD86 and MHC class II molecules are not upregulated in response to LPS or poly (I:C), and TNFα and IL6 release in response to LPS are completely abrogated in the absence of TRIF (Yamamoto et al., 2003). Unfortunately the authors did not discuss the effect TRIF deficiency on poly (I:C)-induced MAPK activation. Indeed, TRIF signaling studies are predominantly focused upon the induction of IRF3-dependent interferon-inducible gene expression. Rip kinases have been shown to function in NF-κB activation downstream of TRIF; but, in contrast to NF-κB, JNK activation is not diminished in the absence of Rip1 (Meylan et al., 2004). The pathways leading to MAPK activation downstream of TRIF remain ill defined; even the requirement of TRIF for TRAF6 has not yet been conclusively established (Gohda et al., 2004). Nonetheless, this report suggests that TRIF-dependent MAPK activation may operate via MLK, and it would be very interesting to investigate this hypothesis further. In initial studies, a cell line would be a more appropriate model for these studies due to the relative ease of biochemical manipulation. It would also be interesting to investigate poly (I:C)-induced MLK3 phosphorylation in the TRIF-deficient cells.

The PI3K pathway may also play an important role in the regulation of MLK signaling. In HepG2 cells, Akt1, a PI3K effector, has been shown to interact with the C terminal portion of MLK3, and phosphorylate MLK3 in this region, which results in inhibition of MLK3-mediated JNK activation (Barthwal et al., 2003). Inhibitor studies have indicated a role for PI3K in DC in a pro-survival and anti-apoptotic capacity, and with regards data presented in this thesis, it is possible that these effects are mediated via inhibition of MLK proteins. Pointedly, H_2O_2-induced fibroblast apoptosis has been associated with a reduction in Akt activity (Taylor et al., 2004), and Akt1 binding to JIP1 has been shown to reduce JNK activation and consequent apoptosis in neurones and this was associated with the disruption of JIP1 binding to MLK3 (Kim et al., 2002). Furthermore, JIP1 binding to Akt1 has been shown to promote Akt1 activation (Kim et al., 2003; Kim et al., 2002). Clearly this could be a form of negative regulation of MLK3. In concert with our observation that MLK is involved in both apoptosis and maturation, there is evidence that Akt-mediated inhibition may also regulate both of these processes. PI3K-deficient DC demonstrate defective IL12 production in response to various stimuli (Fukao et al., 2002). Interestingly, there was also significant enhancement of p38

activation in response to stimuli including LPS in the PI3K-deficient DC. It is clearly an attractive hypothesis that the observed effects of PI3K on p38 activity are mediated via MLK3, and this obviously requires future investigation. In further support of this hypothesis, Akt2 has been shown to negatively regulate the assembly of the POSH-MLK-JNK signaling complex, suggesting that Akt proteins function to negatively regulate MLK signaling in general (Figueroa et al., 2003).

The prototypical mechanism of MLK activation is via release of autoinhibition upon binding to the GTP-associated forms of rac or cdc42 (section 1.3.3.1), and indeed there is evidence, albeit limited, suggesting that this mechanism may apply downstream of TLRs. Stimulation of TLR2 has been shown to induce transient activation of rac1 and cdc42 (Arbibe et al., 2000), and a signaling complex containing TLR2, rac1, and PI3K was demonstrated. Furthermore, LPS-induced activation of p38 and JNK has been shown to be rac1-dependent in Rat-2 fibroblasts (Woo and Kim, 2002).

Important roles for rac and cdc42 in DC have been demonstrated in the regulation of morphology (Jaksits et al., 2004), with the activation of rac and cdc42 being associated with dendrite extension (Swetman et al., 2002). This is consistent with roles for these proteins in the control of morphology in other cell types. The precise mechanism of morphological control downstream of these GTPases is complex, but has not been proposed to require MLKs. It was therefore interesting to discover that treatment of DC with the CEP inhibitor induced a dramatic extension of dendrites by a majority of the cells (figure 5.1), and this process was not mediated by MAPKs. By time-lapse microscopy, DC *in vitro* can be observed to oscillate between dendritic and round morphologies. The cytoskeletal architecture of DC dendrites has been shown to be maintained in part by microtubules (Swetman, submitted for publication), and depolymerisation of microtubules is presumably required for the transition from dendritic to round-shaped DC. MLK proteins have previously been shown to be localised to microtubules (Rasmussen et al., 1998; Nagata et al., 1998; Swenson et al., 2003), and destabilise microtubules during the cell cycle (Swenson et al., 2003; Cha et al., 2005). We have observed an increase in the proportion of dendritic shaped DC upon MLK inhibition, suggesting that MLK may also function in DC to induce microtubule destabilisation. Therefore the accumulation of dendritic shaped cells that we observe upon MLK inhibition probably reflects the equivalent of cell-cycle blockade, whereby round cells extend dendrites that they are unable to retract due to a lack of MLK activity resulting in an accumulation of cells with a dendritic morphology. To investigate whether CEP induces microtubule stabilisation, it would be interesting to compare the sensitivity of CEP-treated versus control DC to the microtubule stabilising agent taxol. Intracellular staining studies to examine the subcellular localisation of cytoskeletal

components and MLK proteins in both round and dendritic DC upon treatment with LPS or CEP would also provide an interesting insight into this issue.

6.3 Limitations and Future Directions

In the preceding chapters, we have proposed a role for JNK in the control of DC apoptosis (chapter 3), and roles for MLK proteins in DC in the regulation of apoptosis (chapter 3), the control of maturation in response to a selection of TLR ligands (chapter 4), and in the induction of increased dendritic morphology (chapter 5). It is important to note, however, that these conclusions are based, in part, on experiments utilising pharmacological inhibitors. A limitation of studies utilising such inhibitors is that it is impossible to be certain that the inhibitor is binding exclusively to the molecule of interest. Indeed, the p38 inhibitor SB203580, which has been studied extensively and used here as a positive control, may also inhibit phosphoinositide-dependent protein kinase 1, and activate Raf-1, at the concentration used in this study (Lali et al., 2000; Kalmes et al., 1999). All of the findings in this thesis therefore need to be confirmed using alternative experimental approaches.

In addition to issues regarding the specificity of the inhibitors used, it is also possible that the TLR agonists used in the study; LPS, poly (I:C), and zymosan are not necessarily TLR-specific agonists. Indeed, poly (I:C) has previously been suggested to stimulate protein kinase R [Diebold], whilst zymosan may activate one or a combination of different C-type lectins, and has recently been shown to agonise Dectin-1 (Rogers et al., 2005). It would be interesting to repeat the experiments of Chapter 4 in TLR-, protein kinase R-, or Dectin-1-deficient DCs to examine the relative contribution of these additional pathways to the effects poly (I:C) and zymosan reported here. Furthermore, the purity of all three stimuli was not examined, and therefore additional receptors may also be stimulated. Experiments with these knockout models would also prove useful in clarifying this issue.

Primary cells such as the monocyte-derived dendritic cells used in this study are difficult to manipulate genetically. Indeed, we have attempted to verify the observations reported in this thesis by expressing mutant MLK3 proteins in DC. However, technical difficulties prevented us from retaining the immature state of the cells following transfection. Furthermore, the process of transfection of human DNA itself has been shown recently to be immunostimulatory in a macrophage-like cell line (Jiang et al., 2004). Biochemical analysis of MLK3 was hampered by technical difficulties in successful immunoprecipitation due to the unavailability of suitable antibodies. This approach will clearly become possible in the future as reagents become available. Therefore, despite their limitations, inhibitors remain a key tool for

studying intracellular signaling in DC. Better methods of genetic manipulation of DC, and better reagents will allow further investigation based on the results of current inhibitor studies.

An obvious approach to verify our observed roles of the JNK and MLK pathways in DC is to repeat the experiments in mice deficient for the appropriate genes. Knockout mice with deletions of both the *Jnk1* and *Jnk2* loci have been reported. Individually, neither of these mutations are lethal and do not result in obvious defects. Mice lacking both JNK1 and JNK2 isoforms, however, do not survive beyond the late embryonic stage. DC from mice lacking either JNK1 or JNK2 have not been examined, and it would be interesting to determine the resistance of these DC to oxidative stress in comparison to wild-type littermate controls, although the lack of a phenotype in these single knockout mice suggests that each isoform is redundant. This "compensatory" mechanism is likely to also operate in the response to oxidative stress and therefore it may be difficult to interpret the results of such studies in DC. The optimal approach would be to engineer mice with a DC-specific deficiency for both JNK isoforms. More recently, MLK3 knockout mice have been generated, and these also demonstrated little phenotypic abnormality (Brancho et al., 2005). Again, this is probably a result of functional reconstitution by other MLK isoforms; and DC were not examined.

An important aspect of pharmacological studies is to correlate the biological and biochemical dose response profile of the inhibitors. An intriguing observation within the data presented here is that the concentration of the CEP inhibitor at which inhibition of hydrogen peroxide-induced DC apoptosis is observed is 0.03μM. In contrast to this, the maximal inhibition of TLR-induced maturation and the greatest dendrite extension are observed at 1μM. It is possible that the effects observed at the higher inhibitor concentration are non-specific, although 1μM CEP is consistent with the concentrations used in most other studies (Ciallella et al., 2005; Hidding et al., 2002). Furthermore, a function for MLK downstream of TLRs is suggested by the observation that TLR ligation induces MLK3 phosphorylation (figure 4.3), and biochemical studies have indicated a role for MLK3 in p38 activation (Kim et al., 2004).

An explanation for the discrepancy in optimal CEP inhibitor concentration is that partial inhibition of JNK (such as is observed at low CEP inhibitor concentrations) may be sufficient to prevent cell death. Such an interpretation is consistent with the data presented in figure 3.9a, in which only partial inhibition of JNK phosphorylation is observed at 0.03μM CEP, despite strong inhibition of apoptosis at this inhibitor concentration (figure 3.10). An alternative, but not mutually exclusive explanation is the possibility that the control of apoptosis and TLR-induced maturation are mediated by different MLK isoforms. We

demonstrated that four different MLK isoforms are expressed in DC (figure 4.2), and three of these are known to be inhibited by the CEP inhibitor (and the fourth, MLK4, has not been tested). The MLK proteins are similar, but not identical. Hence the efficacy of the inhibitor for each protein is likely to differ. It is therefore possible that the CEP inhibitor inhibits the MLK protein involved in the apoptotic pathway with a higher efficiency than the MLK protein activated downstream of the TLRs, for example. Such an interpretation of different MLK isoforms operating upstream of p38 and JNK MAPKs in DC is reinforced by the observation that in figure 3.9, JNK inhibition and p38 inhibition occur at slightly different inhibitor concentrations. This is consistent with a model in which JIP scaffolds, with different MLK isoform affinities, selectively bind p38 or JNK and are used to maintain signal pathway specificity. The idea of different MLK proteins controlling apoptosis and maturation also solves another apparent paradox of the thesis - that MLK is activated by TLR ligands (chapter 4), and induces apoptosis (chapter 3), but TLR ligation does not necessarily result in apoptosis.

6.4 Conclusions

In this study we have demonstrated that JNK activation regulates the apoptosis of DC, and this represents a potentially important mechanism limiting unwanted specific immune responses. Maturation of DC is controlled in part by p38, and the data presented in this thesis indicates that by operating upstream of these MAPKs, MLKs play an important role in the regulation of apoptosis and maturation. We believe that the identification of the capacity of MLK inhibitors to dissociate the functional and morphological maturation of DC makes them a useful tool in future studies dissecting the process of DC maturation. A more detailed examination of the functional implications of MLK inhibition of TLR signaling may lead to the wider use of these drugs in an anti-inflammatory capacity. Furthermore, the identification of proteins globally regulating DC maturation in response to some but not all stimuli indicates a potential avenue for therapeutic intervention in a stimulus-specific manner.

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